EFFECT OF NITRATE ON HUMAN CELL LINES IN CULTURE

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By

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Keywords: nitrates, protein expression, cytotoxicity, HepG2, HEK293, tissue culture

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ABSTRACT

Nitrate is a ubiquitous drinking water contaminant with potential adverse effects on human health. However, little is known about nitrate toxicity at the cellular and molecular level. The purpose of this study was to examine the effects of environmentally relevant concentrations of nitrate on cytotoxicity and protein expression in human cell lines. To determine if tissue-specific responses occurred, a human hepatoma cell line (HepG2) and a human embryonic kidney cell line (HEK293) were used. Both potassium and ammonium salts of nitrate were used to determine salt-specific toxicity. Test concentrations of nitrate varied from 1 µg/L to 5000 mg/L. Cells were exposed to a nitrate salt for 24, 48, or 72 hours and then examined for effects on viability (using the Neutral Red assay) or proliferation (using the BrdU ELISA assay). To determine the effects of nitrate on protein expression, levels of PCNA, Hsp70, Hsc70, and VEGF protein were monitored using Western blotting in HepG2 and HEK293 cells exposed to KNO_3 or NH_4NO_3 for 24 hours.

Nitrate was cytotoxic to both cell types at high concentrations, with EC_{50} values between 1557 mg/L – (approximately) 5852mg/L for viability, and ~2.5 mg/L – 3631 mg/L for proliferation. Several EC_{50} values were not calculable based on the available data, but appeared to be far greater than 5000mg/L. Ammonium nitrate was generally more toxic than potassium nitrate, and increasing exposure time generally resulted in greater toxicity. The HepG2 and HEK293 cells displayed similar responses for most assays, except the 24 hour KNO_3 Neutral Red assay. Here, HEK293 viability increased with increasing KNO_3 concentrations, while HepG2 viability decreased. The reason for this

finding is unknown, but may involve cell-specific homeostatic mechanisms. A hormetic-like effect was observed in both cell types in several of the proliferation assays; the biological significance of this effect remains unknown.

No significant changes in protein expression were observed under these experimental conditions. Some subtle trends were present, such as a slight increase in Hsp70 expression with increasing nitrate concentration in both cell types. In HepG2 cells, PCNA expression increased slightly with increasing nitrate concentrations; however, the opposite effect was observed in HEK293 cells. This may be due to transcriptional or translational regulation.

In summary, environmentally relevant concentrations of nitrate did not appear to evoke significant cytotoxicity or changes in protein expression. Cell viability and proliferation effects were observed at higher concentrations of nitrate. Private water supplies may contain nitrate concentrations above the EC_{50} values in these experiments. More research is required to determine if this poses a direct threat to human health.

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Finally, I would like to thank my sweetheart, Wade Lahoda, for being so patient, loving, and understanding during this degree. He always knew the right time to suggest a trip for ice cream or a big bear hug. Thanks, sweetie.

DEDICATION

I dedicate this thesis to all my families,
whether we're related by blood or by love,
and especially to the memories of
Kent Zuehlke
and
Donnalee Zuehlke
I miss you and I love you.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
µL	microlitres
µm	micrometres
ANOVA	analysis of variance
APS	ammonium persulphate
ATP	adenosine triphosphate
BrdU	5-bromo-2’-deoxyuridine
C. dubia	*Ceriodaphnia dubia*
CAS	Chemical Abstracts Service
CCME	Canadian Council of Ministers of the Environment
cDNA	complementary DNA
CH_3COO^-	acetate ion
CH_3COOK	potassium acetate
CH_3COONH_4	ammonium acetate
cm^2	centimetres squared
CNS	central nervous system
CO_2	carbon dioxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EC_{50}	concentration which causes 50% maximal effect
ECF	extracellular fluid
ED	ecodistrict

EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
GI	gastrointestinal
h	hour
H^+	hydrogen ion
H_2O	water
H_2SO_4	sulphuric acid
HCl	hydrochloric acid
HEK293	human embryonic kidney epithelial cell line
HepG2	human hepatocellular liver carcinoma cell line
Hsc70	heat shock cognate protein 70
Hsp70	heat shock protein 70
IC_{50}	concentration which causes 50% inhibition
ICD	International Classification of Diseases
IDDM	insulin-dependent diabetes mellitus
ILO	intensive livestock operation
ITRC	Interstate Technology Regulatory Cooperation
IUGR	intrauterine growth restriction
K^+	potassium ion
kDa	kilodalton

KNO_3	potassium nitrate
L	litre
LD_{50}	lethal dose for 50% of experimental subjects
LOEC	lowest observed effect concentration
M	moles per litre
MAC	Maximum Acceptable Concentration
MCL	Maximum Contaminant Limit
MEM	minimum essential media
MeOH	methanol
MetHb	methemoglobin
$MgCl_2$	magnesium chloride
mL	millilitres
MMWR	Morbidity and Mortality Weekly Report
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight
N_2	dinitrogen gas
Na^+	sodium ion
Na_2HPO_4	sodium hydrogen phosphate
NaH_2PO_4	sodium phosphate monobasic
$NaNO_3$	sodium nitrate
NH_3	ammonia

NH_4^+	ammonium ion
NH_4NO_3	ammonium nitrate
NHL	Non-Hodgkin Lymphoma
nm	nanometres
NO	nitric oxide
$NO^{\cdot}$	nitric oxide radical
NO_2^-	nitrite
$NO_2^{\cdot}$	nitrogen dioxide radical
NO_3^-	nitrate
NO_3-N	nitrate-nitrogen
NOAEL	no observed adverse effect level
NRC	National Research Council
NTD	neural tube defect
O_2	oxygen gas
$ONOO^-$	peroxynitrite
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline + Tris
PCNA	proliferating cell nuclear antigen
PMSF	phenylmethylsulfonyl fluoride
POD	peroxidase
RNA	ribonucleic acid
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate

SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
TEMED	tetramethylethylenediamine
TSH	thyroid stimulating hormone
USEPA	United States Environmental Protection Agency
V	volt
VEGF	vascular endothelial growth factor
WHO	World Health Organization
WSZ	water supply zone

CHAPTER 1: INTRODUCTION

1.1 Sources of nitrate

Nitrates (NO_3^-) are ubiquitous drinking water contaminants, found in virtually every water supply in the world. An important component of the nitrogen cycle, nitrates serve as a biologically available nitrogen source for plants. Historically, the nitrogen cycle has maintained a balance of nitrogenous compounds and ions in the environment; however, with increasing human activity, population, and the use of nitrogenous fertilizers, nitrate input has exceeded the nitrate uptake capacity of the biosphere (WHO, 2004). Since nitrate is a relatively stable ion, and highly mobile in water and soil, it can easily enter the water table (Rasiah *et al.*, 2005), or pollute surface waters via run-off from contaminated soil. This has resulted in increased nitrate concentrations in global groundwater supplies over the past 20 years (WHO, 2004).

Determining the source of nitrate contamination, however, is difficult, since nitrate can arise from natural or anthropogenic sources. Natural sources include geological contributions (fixed ammonium in rocks, salt slicks) and soil organic matter (leaf litter, organic detritus) (Henry, 1995). Anthropogenic sources include the spreading of animal manure for agricultural purposes, the use of inorganic nitrogenous fertilizers, leaching from poorly-designed landfills, improper sewage disposal, and atmospheric pollution from combustion engines or industrial energy production (WHO, 2004). Over-fertilizing of agricultural land, particularly when animal manure and inorganic fertilizers are used

in combination, can readily overload the nitrate uptake capacity of the crops, leading to an excess of nitrate that may enter the groundwater supply (WHO, 2004). The burgeoning populations in developing countries, combined with the lack of sanitary sewage systems, water treatment facilities, or regulation of discharging substances into the environment, has resulted in severe groundwater nitrate pollution in many regions of Asia, Africa, and Mexico (WHO, 2004).

1.2 Nitrate and drinking water

Due to the ubiquity of nitrate, it is expected that humans will be exposed to nitrates through diet and drinking water. Although Canadian public water supplies generally contain a low level of nitrate (5 mg/L NO_3^-), private wells may have much higher nitrate concentrations, potentially exceeding the Maximum Acceptable Concentration (MAC) of 45 mg/L NO_3^- (Health Canada, 1992). Wells that are poorly constructed or contaminated with human or animal waste may have dangerously high nitrate concentrations (WHO, 2004).

The relative contribution of drinking water to daily nitrate intake depends on the quality of the water source as well as diet. For the majority of Americans, the major nitrate source is diet, with 86% of daily intake coming from vegetables; drinking water makes up about 3% (NRC, 1995). However, in individuals who regularly consume nitrate-rich drinking water, up to 69% of the daily nitrate consumption may be attributed to drinking water. Consumption of nitrate-rich drinking water may result in a total daily nitrate intake of 233 mg, compared to an intake of 75 mg with a low-nitrate water source (NRC, 1995). A high proportion of nitrate intake from drinking water is problematic because

ingested nitrate may contribute to the formation of carcinogenic N-nitroso compounds. While vegetables contribute significantly to nitrate intake, they also contain anti-oxidants and vitamins that may reduce cancer risk. Drinking water does not contain these substances, so the risk of adverse effects may be elevated (WHO, 2004). It is unclear whether the nitrate in vegetables is present in the flesh or the water content, but given the high water-solubility of nitrate and elevated nitrate levels in vegetables that have high water content (Thomson *et al*., 2007), it is likely that at least some of the nitrate is present in the water. However, as previously stated, the nutritional benefits of vegetables generally outweigh the risk posed by elevated nitrate concentrations.

1.3 Nitrate and health effects

While nitrates play an important role in the biosphere as integral components of the nitrogen cycle, excessive nitrate concentrations in drinking water may have serious implications for human health. The toxic effects of nitrate exposure are most commonly associated with acute, high-dose exposure. Methemoglobinemia, a condition arising from the conversion of hemoglobin to a form that can no longer carry oxygen (methemoglobin, or MetHb), is the best-known manifestation of drinking-water nitrate toxicity. Methemoglobinemia can affect all ages, but clinical manifestation is almost exclusively restricted to young infants, often less than 60 days old (Shearer *et al.*, 1972). The current Maximum Acceptable Concentration (MAC) of nitrate in drinking water (45 mg/L NO_3^-) is designed to avoid cases of infant methemoglobinemia (Health Canada, 1992). Fatal cases of infantile methemoglobinemia linked to nitrate-contaminated drinking water exposure have occurred in Saskatchewan (Robertson & Riddell, 1949) and, more recently, in the United States (Johnson *et al*., 1987).

Many epidemiological studies have investigated the role of drinking water nitrate in the development of chronic health conditions. Several cancers are suspected to have an association with drinking water nitrate concentration, including Non-Hodgkin Lymphoma (Ward *et al*., 2006; Ward *et al*., 1996; Cocco *et al*., 2003; Freedman *et al*., 2000; Law *et al*., 1999), gastrointestinal cancer (Yang *et al*., 1998; Barrett *et al*., 1998; De Roos *et al*, 2003), brain cancer (Barrett *et al*., 1998; Ward *et al*., 2005), pancreatic cancer (Coss *et al*., 2004), urological cancer (Ward *et al*., 2003; Zeegers *et al*., 2006; Volkmer *et al*., 2005), and others (Weyer *et al*., 2001). Nitrate may also contribute to the development of Type 1 insulin-dependent diabetes (Parslow *et al.*, 1997; van Maanen *et al*., 2000; Zhao *et al*., 2001; Moltchanova *et al*., 2004), thyroid disorders (Tajtáková *et al*., 2006; van Maanen *et al*., 1994), and teratogenic or reproductive effects (Brender *et al*., 2004; Dorsch *et al*, 1984; MMWR, 1996; Frecker & Frasier, 1987; Arbuckle *et al*., 1988; Bukowski *et al*., 2001; Bunin *et al*., 1994). Despite the large number of studies conducted, results are inconsistent, with evidence both supporting and refuting a role for drinking water nitrate in disease development. More research is required before the contribution of drinking water nitrate to adverse health effects is fully known.

A previous examination of the effect of nitrate on gene expression was conducted by Bharadwaj *et al*. (2005). A cDNA microarray system was used to screen 13744 genes in HepG2 cells exposed to potassium nitrate for 24 hours. Environmentally relevant concentrations of 3 mg/L, 45 mg/L, 150 mg/L and 500 mg/L KNO_3 were used. This study found that 138 genes experienced at least a two-fold change in expression, with an equal proportion of up- and down-regulated genes. Some of the genes significantly affected by nitrate exposure control intracellular processes such as cell cycle control,

DNA repair, and responses to stress. Because of the wide range of processes potentially affected at environmentally relevant nitrate concentrations, Bharadwaj *et al*. (2005) emphasize the urgent need for more research on this topic to fully understand the potential human health effects.

In summary, the presence of nitrates in drinking water may constitute a human health hazard. Because individuals may be constantly exposed to nitrate-contaminated drinking water, there is a potential for chronic toxicity. Several epidemiological studies have attempted to determine the relationship between drinking water nitrate consumption and a variety of chronic health conditions, such as cancer, diabetes, and thyroid problems. However, these studies are largely inconclusive and hampered by issues regarding sample size and the heterogeniety of nitrate concentrations in water supplies. While further epidemiological studies are necessary, an investigation into the cellular and molecular effects of environmentally relevant nitrate concentrations on human cells is also required. This study will attempt to bridge the knowledge gap of the effects of nitrate on human cells at the cellular and molecular level through examination of cell viability, proliferation, and protein expression.

1.4 Hypotheses

Based on previous research and available evidence, the hypotheses of this project are as follows:

I. Nitrate exposure will produce cytotoxic effects, defined as a reduction in cell viability and/or proliferation, in exposed populations of HepG2 and HEK293 human cell lines. The severity of the cytotoxic response is expected to increase with nitrate

concentration and exposure time. These effects may occur above or below nitrate concentrations typically found in drinking water.

II. The expression patterns of specific cellular proteins in HepG2 cells after exposure to environmentally relevant concentrations of nitrate will resemble the gene expression pattern elucidated in Bharadwaj *et al*. (2005). In other words, the expression pattern changes observed at the level of translation (in this study) will be similar to those observed at the level of transcription (in Bharadwaj *et al*., 2005). Specifically, PCNA and Hsp70 expression are expected to increase with increasing nitrate concentrations, while VEGF is expected to decrease with increasing nitrate concentrations. No change in expression is anticipated for HSC70. Since no expression pattern has been previously determined for HEK293 cells, they are expected to respond in a manner similar to the HepG2 cell line.

III. Significant cytotoxic effects are not expected to be observed in HepG2 and HEK293 cells exposed to nitrate concentrations below the current drinking water Maximum Acceptable Concentration (MAC) of 45 mg/L NO_3^-. However, changes in protein expression are expected to be observed in cells exposed to nitrate at concentrations both above and below the current MAC.

1.5 Objectives

The specific objectives of this research are as follows:

I. To determine the concentration of potassium and ammonium nitrate that results in significant decreases in cell viability in HepG2 and HEK293 cell lines at 24, 48, and 72 hour exposure times using the Neutral Red cytotoxicity assay.

II. To determine the concentration of potassium and ammonium nitrate that results in significant decreases in cell proliferation in HepG2 and HEK293 cell lines at 24 and 72 hour exposure times using the BrdU ELISA cytotoxicity assay.

III. To determine relative expression levels of specific proteins in HepG2 and HEK293 cells exposed to potassium and ammonium nitrate at concentrations both above and below the current MAC of 45 mg/L NO_3^- at 24 hour exposure times using Western blotting.

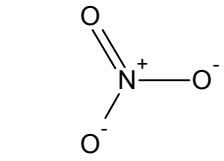
CHAPTER 2: LITERATURE REVIEW

2.1 Chemical properties of nitrate

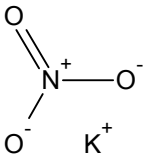
Nitrate (NO_3^-) is a form of nitrogen commonly found in the environment. Nitrate may arise through natural processes, or through anthropogenic outputs. While nitrate does play a vital role in the nitrogen cycle and other natural processes, human activities are producing excessive nitrate loads which exceed the capacity of the surrounding environment (WHO, 2004).

The structures of nitrate and some of its salts are displayed in Figure 2.1. Nitrate and its salts are very water-soluble. Nitrate may move freely with soil water, and, unlike ammonium, is generally not bound to the soil rock matrix (WHO, 2004). This may lead to leaching, a process by which solutes, such as nitrate, are dissolved and removed from soil by water. Nitrate-contaminated leachate can easily enter ground or surface water and cause further contamination.

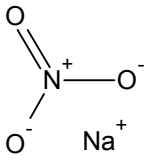
Nitrate is closely related to another nitrogenous compound called nitrite (NO_2^-). Nitrite is also found in the environment, but is much less stable in oxygenated environments than nitrate. Thus, nitrate is normally present in water at much greater concentrations than nitrite. Because nitrate is a stable ion, many nitrogenous compounds, including nitrite, are converted to nitrate in the environment. Nitrate itself appears to be less toxic than nitrite, but may be converted into nitrite *in vivo* (see section 2.8), or under



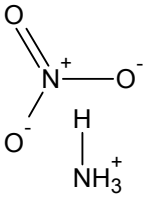
Nitrate ion
MW: 62.005g/mol
CAS#: 14797-55-8



Potassium nitrate
MW: 101.09g/mol
CAS# : 7757-79-1



Sodium nitrate
MW: 84.995g/mol
CAS# : 7631-99-4



Ammonium nitrate
MW: 80.043g/mol
CAS# : 6484-52-2

Figure 2.1: Structure and characteristics of nitrate and selected salts

anaerobic conditions. Both nitrate and nitrite must be considered when examining water quality (Health Canada, 1992).

It is important to note that there are two different systems of measurement for nitrate. These systems are not equivalent and confusion may arise from the application of an incorrect system. The World Health Organization and Health Canada use the nitrate system of mg/L as NO_3^- to measure nitrate concentrations. However, the United States Environmental Protection Agency (USEPA) uses the mg/L NO_3-N (nitrate-nitrogen) system for the measurement of nitrate. While the regulatory limits of 45 mg/L NO_3^- (Health Canada) and 10 mg/L NO_3-N (USEPA) look different, when the appropriate conversion factors are applied they are in fact virtually the same. To convert from nitrate-nitrogen to nitrate, the following equation is used (Bruning-Fann & Kaneene, 1993):

$$\text{Nitrate-nitrogen } (NO_3\text{-N}) \times 4.45 = \text{Nitrate } (NO_3^-) \quad (2.1)$$

Caution must be used when interpreting the results of publications using measurements of nitrate, as some authors are not aware of the difference between systems or do not specify which system is used in their calculations.

In the environment, nitrate plays an important role in the nitrogen cycle (Figure 2.2). It is the primary accessible source of nitrogen for plants, which use nitrate to synthesize organic nitrogenous compounds, such as proteins (WHO, 2003). Nitrate is formed though the breakdown of nitrogenous compounds by bacteria, a process called

nitrification, or through the oxidation of atmospheric nitrogen by lightning (Coyne & Thompson, 2006). As shown by Figure 2.2, several processes occur in air, soil, and groundwater which involve the interchange of nitrogenous compounds (USEPA, 1993).

Many industrial uses have been found for nitrate and its salts. The reactive properties of ammonium nitrate are useful in explosives, while the antimicrobial action of sodium nitrate and sodium nitrite is used as a preservative for cured meats. Potassium nitrate is used in glassmaking and inorganic fertilizers. Nitrate-based pharmaceuticals dilate blood vessels and are used in the treatment of angina and hypertension. Nitrate-based inorganic fertilizers provide nitrogen to growing plants, a valuable property in areas where soil is nutrient-poor. While it is certain that the use of nitrate-based products has proven beneficial, the over-use of nitrate is contributing to the degradation of global drinking water (WHO, 2004).

The chemical characteristics of nitrate have made it valuable for both environmental and industrial uses. Although the high water-solubility of nitrate renders it invaluable in the nitrogen cycle, it also contributes to the contamination of drinking water supplies. The over-use or misuse of nitrate in human activities has exacerbated this problem. Careful monitoring of nitrate concentrations, as well as proper nitrate management, will ensure that the beneficial characteristics of nitrate will continue to aid humanity.

2.2 Environmental and anthropogenic sources of nitrate

Nitrate is a unique substance in that it can arise from both natural and anthropogenic sources. This quality, along with the vast number of potential sources, can make the

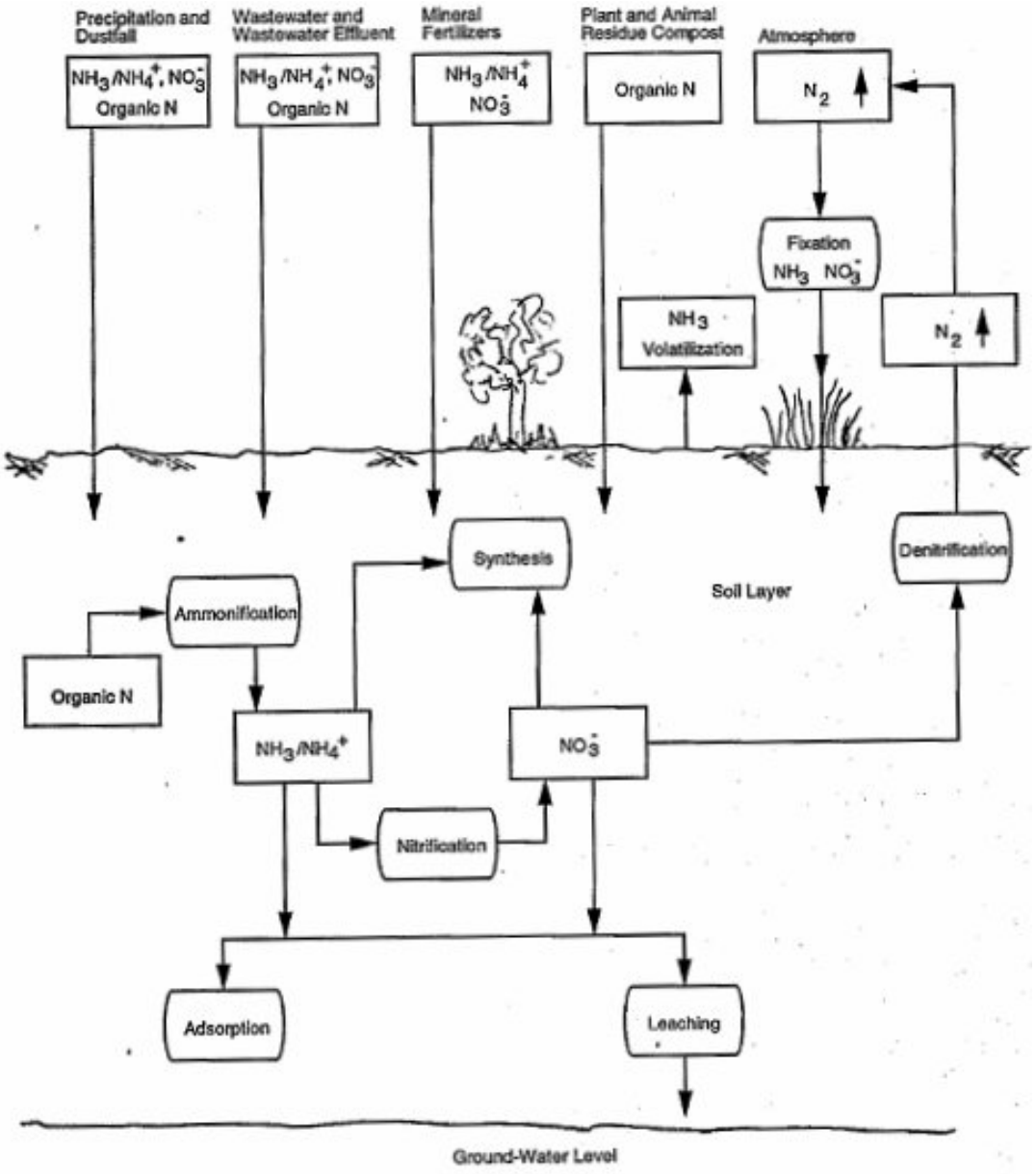


Figure 2.2: The nitrogen cycle in air, soil, and groundwater (USEPA, 1993)

source of nitrate difficult to pinpoint. While some nitrate sources are common to all areas, others may be more common to agricultural areas, or economically developing regions. Achieving a balance of nitrate flow is important in order to prevent contamination of drinking water supplies.

Nitrate is an integral part of the nitrogen cycle and is produced by several natural processes. The aerobic decomposition of organic material, such as waste products, manure, and non-living organisms, produces nitrate (WHO, 2004). Organic soil matter and leaf litter may also serve as a nitrate source (Henry, 1995). Nitrate may be produced from ammonia via microbial nitrification by *Nitrosomonas* (1) and *Nitrobacter* (2) bacteria as follows (WHO, 2004; Khin & Annachhatre, 2004):

(1) $NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2H^+$

(2) $NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$

Geological formations may also serve as natural nitrate sources. Examples of these include natural sodium nitrate deposits, salt slicks, and weathered till (Henry, 1995).

Agriculture is often perceived as a large contributor to anthropogenic nitrate pollution. Both animal manure and inorganic fertilizers contain nitrate, which has the potential to leach and enter water sources. Overfertilizing of agricultural land, particularly when animal manure and inorganic fertilizers are used in combination, can readily overload the nitrate uptake capacity of the crops, leading to an excess of nitrate that may enter the groundwater supply (WHO, 2004). The prevalence of Intensive Livestock Operations

(ILOs) in Western Canada may also contribute to nitrate pollution through the large volumes of animal manure produced. However, legislation has been implemented to regulate ILO waste management practices and therefore prevent groundwater contamination (Thompson, 2001).

Non-agricultural anthropogenic nitrate sources may also significantly contribute to nitrate contamination of water supplies. Poorly-designed landfills may leach nitrate into groundwater (WHO, 2004); this may be a serious concern for some Canadian First Nations communities, who have traditionally suffered from insufficient waste-disposal facilities (Bharadwaj *et al*., 2006). Improper disposal of sewage, and inadequate or leaky sewage systems may pollute drinking water sources (WHO, 2004). Atmospheric pollution from combustion engines or industrial energy production can contain nitrate which may enter water systems (WHO, 2004). The burgeoning populations in developing countries, combined with the lack of sanitary sewage systems, water treatment facilities, or regulation of discharging substances into the environment, has resulted in severe groundwater nitrate pollution in many regions of Asia, Africa, and Mexico (WHO, 2004).

Potential sources of nitrate in urban areas may include leaching from contaminated land, industrial spills, and perturbation of land for house-building (Wakida & Lerner, 2005). Excavation results in the mixing of soil, increasing aeration and the availability of new nitrogen sources; this may increase the amount of nitrate produced by soil biota. Land used for the production or testing of explosive substances may also be a significant source of nitrate, since many explosives are produced from nitrate or release nitrate

upon detonation. Due to the large number of potential nitrate sources in a relatively smaller area, nitrate pollution in urban areas may be similar to that found in rural agricultural areas (Wakida & Lerner, 2005).

It is important to note that nitrate exposure may also arise from less obvious anthropogenic sources. Occupational exposure to nitrate is possible in industries or activities which involve prolonged contact with nitrate sources. These include the production of fertilizer, ink, and explosives; lithography; petroleum refining; and gunsmithing (Seifert, 2004). Treatment with nitrate-based pharmaceuticals is another possible route of exposure; these compounds are often prescribed for the relief of angina pectoris and to reduce cardiac workload in congestive heart failure and hypertension (Siefert, 2004). Silver nitrate, used as an antimicrobial agent in the treatment of burn victims, can induce toxicity in susceptible patients (Chou *et al*., 1999). Several accidental intoxications with nitrate have been reported in the literature after consumption of contaminated herbal medicine (Chui *et al*., 2005), medical cooling pack contents (Challoner & McCarron, 1988), and nitrate-contaminated ice (Brunato *et al*., 2003).

Potential nitrate sources are varied and may arise from natural or human activity. Natural sources include decomposing organic matter and geological formations; anthropogenic sources include agricultural runoff, sewage, industrial activity, and pharmaceuticals. Nitrate concentrations in a given area are dependent on both natural and anthropogenic sources, and may vary based on the local ecosystem and degree of human activity. Both developed and developing nations may experience nitrate

contamination, but the sources responsible may not be the same for each. Proper management of human activity and knowledge of natural sources is essential to minimize potentially harmful drinking water nitrate contamination.

2.2 Nitrate and drinking water contamination

Because nitrate is so ubiquitous, it is expected that the vast majority of water systems will contain at least some nitrate. Generally, levels of background nitrate in pristine water systems are low – up to 10 mg/L NO_3^- for groundwater and 5 mg/L NO_3^- for surface water. Values above these concentrations may be indicative of an external nitrate source (WHO, 2004). Due to human input, nitrate concentrations in both surface and ground water have increased in many countries since the mid 1950s (WHO, 2004).

Monitoring groundwater quality is especially crucial because of its importance as a drinking water source. In Canada, an estimated 30% of the population (equivalent to 8.9 million people) rely on groundwater for their domestic water needs (including drinking water). The proportion of the population reliant on groundwater ranges from 100% in Prince Edward Island to 23.1% in Alberta, with Saskatchewan at 42.8% (Environment Canada, 2004). In Europe, drinking water consists almost exclusively of groundwater in several countries (Chilton, 1996). Surface water, such as lakes and rivers, is also an important source of drinking water for many populations (Meybeck *et al*., 1996; Thomas *et al*., 1996).

Agricultural practices can greatly influence the potential for drinking water nitrate contamination. The development of intensive livestock operations (ILOs), and the

subsequent manure produced, proportionately increases the amount of nitrate available to contaminate drinking water (Burkart & Stoner, 2002). Krapac *et al* (2002) have shown that swine manure pits may adversely affect local groundwater quality, but the significance of this finding is questioned. The application and composition of fertilizer will also have an impact on water contamination; over-use of fertilizers, particularly when inorganic fertilizers are used with animal manure, can produce excess nitrate which may be leached into groundwater (WHO, 2004). Inappropriate timing of fertilizer application may also lead to drinking water contamination. Common application times in the fall and spring coincide with periods of low crop and soil biota activity. Less nitrate is taken up or transformed during these periods, so more is potentially available to leach into groundwater. Higher rainfall during these periods can also increase nitrate leaching (Burkart & Stoner, 2002).

Crop choice and cultivation may also increase the risk of drinking water contamination. Some crops, such as corn, potatoes, and vegetables, have high nitrogen requirements and often require irrigation. This may lead to water contamination through the large amount of fertilizer required, and the tendency of irrigation to increase nitrate leaching potential (Burkart & Stoner, 2002). A study by Burkart and Stoner (2002) found significant increases in groundwater nitrate concentrations in agricultural areas producing cattle and grains; corn, soybeans, and hogs; or small grains. The same study also found that groundwater nitrate concentrations in irrigated agricultural areas were significantly higher than those of unirrigated agricultural land (Burkart & Stoner, 2002).

Aquifer characteristics may also influence susceptibility to nitrate contamination. Certain types of aquifers, such as unconsolidated and alluvial aquifers, are more susceptible than carbonate aquifers (Burkart & Stoner, 2002). However, the characteristics of the overlying soil can also influence nitrate concentrations. Well-drained, permeable soil increases the ability of nitrate to leach into the aquifer (Burkart & Stoner, 2002). In highly-irrigated areas, shallow unconfined aquifers showed significantly higher nitrate concentrations than those in non-irrigated regions, regardless of overlying soil type. Aquifers with these characteristics are thought to be the most susceptible to agriculture-related nitrate contamination (Burkart & Stoner, 2002). Nolan and Hitt (2006) add high regional nitrogen application, the presence of fractured rock formations, and lack of attenuation processes to their criteria for aquifers at high risk of nitrate contamination.

Remediation of nitrate contamination may be essential to ensure the availability of safe drinking water. The source of nitrate (inorganic fertilizer, manure, sewage, etc.) may be determined by examining the isotopic signatures of the nitrogen and other elements in each suspected source, with a comparison to the species present in the water. However, this may not always be possible since biological modification may change the isotope signature (Widory *et al*., 2004). If the source is properly identified, steps may be taken to lessen the resulting nitrate contamination. Strategies at the level of water treatment include ion-exchange denitrification, where nitrate is exchanged for chloride; biological denitrification, where bacteria reduce nitrate to nitrogen gas; and reverse osmosis, where water is forced through a membrane which removes nitrate (WHO, 2004; Darbi *et al*., 2003). Additional nitrate removal technologies, such as aboveground denitrification and

permeable reactive barriers, are currently undergoing development (ITRC, 2000). The choice of an appropriate treatment strategy will depend on water system characteristics, as different systems are better suited to different conditions.

Nitrate contamination of drinking water may occur naturally, but the recent increase in nitrate concentrations is likely due to human activity. Both surface and ground waters serve as important sources of drinking water both in Canada and around the world, and must be protected. Although local practices such as agriculture have the potential to release large amounts of nitrate into drinking water sources, individual hydrogeologic characteristics of each source will also determine the susceptibility to nitrate contamination. While effective methods of remediating or reducing nitrate contamination exist, alterations in management practices and awareness of local hydrogeologic properties may help prevent contamination of drinking water supplies.

2.4 Environmental effects of nitrate

Although existing regulatory limits are primarily designed to avoid acute human toxicity, nitrate has also been shown to have harmful effects on the environment. While nitrates do occur naturally, human activities have greatly increased the amount of nitrate present in the environment. The high solubility of nitrate can result in contamination of local sources of water, through transport in run-off or leaching (WHO, 2004). This can potentially lead to toxicity for many organisms, particularly those inhabiting aquatic environments. Existing evidence suggests that nitrate may have harmful effects on amphibians, aquatic invertebrates, and fish species.

Several researchers have found evidence that environmentally relevant nitrate concentrations have harmful effects on amphibians. Rouse *et al*. (1999) found that nitrate concentrations below the regulatory limit of 10 mg/L NO_3-N can cause physical and behavioural abnormalities in young and adult frogs. Animals in earlier developmental stages appeared to be more sensitive than more mature individuals (Rouse *et al*, 1999). These effects may be due in part to the development of methemoglobin in the affected animals (Huey & Beitinger, 1980; Hecnar, 1995), which, as in humans, prevents efficient carriage of oxygen throughout the body. Amphibian sexual differentiation may also be affected by nitrate exposure; Orton *et al*. (2006) observed a significantly higher ratio of female to male frogs in tadpoles exposed to 10 mg/L NO_3-N, with or without concurrent atrazine exposure. This may suggest that exposure to nitrate favours the development of female frogs compared to that of male frogs. Premature gonadal development was also noted in the animals, which may have been due to a local endocrine effect in the gonad itself. This finding is troublesome because it indicates that nitrate may interact with other agrochemicals to produce adverse effects, even at concentrations allowable by law.

Other aquatic species may also be adversely affected by nitrate exposure. Environmentally relevant nitrate concentrations were shown to negatively affect reproduction in *Ceriodaphnia dubia*, an aquatic invertebrate, after a 7-day exposure (Scott & Crunkilton, 2000). Chronic nitrate exposure was also found to have a greater-than-expected adverse effect on *C. dubia* reproduction in a study performed by Dave & Nilsson (2005). The nitrate concentrations which evoked this effect were above the MAC but were still environmentally relevant, leading the authors to conclude that “the

chronic hazard of nitrate could be almost as high as that of ammonia" (Dave & Nilsson, 2005). Fish species, especially individuals in early life stages, are also sensitive to nitrate. The 30-day Lowest Observed Effect Concentration (LOEC) for some salmonid species has been shown to occur at nitrate concentrations below the MAC (Kincheloe *et al*., 1979; Camargo *et al*., 2005). These studies demonstrate that a wide variety of aquatic organisms are sensitive to the effects of nitrate exposure, and may be harmed at environmentally relevant concentrations.

Potential protective measures for aquatic life include bans on fertilizer application when vulnerable developmental stages are present or when the potential for runoff is high (Ortiz-Santaliestra *et al*., 2006). The Canadian Council of Ministers of the Environment (CCME, 2006) has introduced an interim freshwater guideline of 13 mg/L NO_3 (2.9 mg/L NO_3-N) specifically for the protection of aquatic organisms. The USEPA does not have such a guideline (USEPA, 1986), but a regulatory limit of 2 mg/L NO_3-N has been encouraged by Camargo *et al*. (2005). While changes to agricultural management practices or regulatory limits may help protect aquatic organisms from nitrate toxicity, it is important to ensure that they are attainable as well as prudent.

Despite significant intra- and inter-species variation, nitrate contamination appears to be a widespread threat to aquatic ecosystems. Several studies have demonstrated the increased susceptibility of early life stages of amphibians, aquatic invertebrates, and fish to nitrate. Toxicity for these organisms may occur at concentrations below the current regulatory limit for drinking water, and may be augmented by exposure to other water contaminants, such as agrochemicals. Nitrate may contribute significantly to chronic

toxicity experienced by aquatic organisms, but more research is necessary to fully elucidate this role. Changes in land management practices or regulatory limits have been proposed to protect aquatic organisms from the harmful effects of nitrate, but the effectiveness of these approaches remains to be seen.

2.5 Human toxicity of drinking water nitrate

2.5.1 Nitrate and methemoglobinemia

The ability of nitrate to produce acute methemoglobinemia, particularly in infants, is the best-known manifestation of nitrate toxicity. The threat of infantile methemoglobinemia is the rationale for the current drinking water standards of 45 mg/L NO_3^- in Canada (Health Canada, 1992) and 10 mg/L NO_3-N in the United States (USEPA, 2006). Briefly, nitrate ingestion induces methemoglobinemia after conversion to nitrite in the body. This may occur through secretion of plasma nitrate into the oral cavity, with subsequent reduction to nitrite by oral flora, or through reduction by gut bacteria (Health Canada, 1992). Nitrite then causes the oxidation of the ferrous (Fe^{2+}) iron in hemoglobin to the ferric (Fe^{3+}) form, which converts the hemoglobin to methemoglobin. Methemoglobin cannot deliver oxygen to tissues, causing hypoxia and cyanosis. Infants appear to be more susceptible for several reasons, including the presence of fetal hemoglobin, which is more easily oxidized; low levels of methemoglobin reductase, which can convert the methemoglobin back to its normal form; a high gastric pH which may facilitate the growth of nitrate-reducing bacteria; and a large intake of fluid relative to body weight (WHO, 2004; Fewtrell, 2004). (A more detailed description of the mechanisms of nitrate toxicity may be found in section 2.8.)

The first report of drinking water nitrate inducing infantile methemoglobinemia was an article by Comly (1945), which detailed two cases of acute methemoglobinemia in two female infants who had been fed formula reconstituted with nitrate-contaminated well water. Both wells greatly exceeded the regulatory limit of 10 mg/L NO_3-N (at 140 mg/L and 90 mg/L NO_3-N), and one well was also found to be bacteriologically unsafe. The infants, aged 2 weeks and 1 month, presented with cyanosis but rapidly improved upon administration of methylene blue. No recurrences were observed once the use of well water was discontinued.

In an attempt to determine if elevated nitrate levels in drinking water affected infant methemoglobin levels on a sub-clinical basis, Shearer *et al*. (1972) examined 256 1-6 month-old babies between 1967-1968. The water source used by the household was tested, and the nitrate intake of each infant was calculated. A blood test for methemoglobin was performed on each infant, and parents were encouraged to bring their children back for follow-up at 2, 3, 4, and 6 months of age. Only 117 infants were examined more than once. Using the data from these infants, Shearer *et al*. discovered that younger infants tended to have higher levels of methemoglobin compared to older infants, independent of the water supply used. This trend was observed even in breast-fed babies. Babies with minor illnesses, especially diarrhea, had higher methemoglobin levels compared to healthy children. However, no consistent or significant correlation was found between methemoglobin level and nitrate intake in the study population.

A case of fatal infant methemoglobinemia was reported in the United States in 1986; this appears to be the latest reported fatality in current literature (Johnson *et al*., 1987).

An 8 week old South Dakota female infant died in 1986 after experiencing a severe cyanotic episode induced by the consumption of formula reconstituted with severely contaminated well water. Upon analysis, it was found that the nitrate concentration of the water was 150 mg/L NO_3-N. More recent reports of non-fatal infant methemoglobinemia in the United States have been reported in Knobeloch *et al*. (2000). In June of 1998, a six-month old male infant experienced cyanosis after drinking formula which had been reconstituted with nitrate-contaminated well water. Tests revealed a nitrate concentration of 22.9 mg/L NO_3-N. The cyanosis resolved after discontinuation of the well water and did not return. In April of 1999, a three-week old female infant experienced severe cyanotic episodes after drinking well-water reconstituted formula. The severity of the cyanosis necessitated transport by helicopter to a regional medical centre. Upon administration of methylene blue, her condition improved rapidly. Analysis of the well water showed a nitrate concentration of 27.4 mg/L NO_3-N and contamination with *E. coli* bacteria. The same authors (Knobeloch *et al*., 2000) performed a search of the national death certificate database and discovered 6 infant deaths attributable to methemoglobinemia in the United States from 1979-1996. Because 5 of these 6 deaths are not reported in the literature, it is not known how many of these cases were due to contaminated drinking water consumption. The one case reported in the literature is discussed above (Johnson *et al*., 1987).

Although many case studies describing drinking water-induced methemoglobinemia exist, some researchers regard nitrate as a “co-factor” or aggravating factor, rather than a primary cause (Fewtrell, 2004). Some researchers, such as Avery (1999; 2001) believe that pre-existing inflammatory conditions cause oxidative stress, creating

methemoglobinemia independent of exogenous nitrate exposure. Elevated nitrate levels are observed in affected infants due to endogenous production (e.g. as a breakdown product of nitric oxide), rather than exogenous sources. However, the validity of this argument is hotly debated (Knobeloch *et al*., 2000; Knobeloch & Anderson, 2001). Based on a review of the literature, it appears that there is sufficient evidence for a role of drinking water nitrate in the development of methemoglobinemia. Although there is no standard level of nitrate exposure which may induce infant methemoglobinemia, the regulatory limit has been set at 45 mg/L NO_3^- because this value corresponds to the NOAEL for nitrate. Methemoglobinemia may occur at concentrations above this limit. However, other factors, such as inflammatory conditions, may also play a role in its development.

2.5.2 Nitrate and cancer

2.5.2.1 Non-Hodgkin Lymphoma

Several studies have examined a potential link between drinking water nitrate concentrations and the development of Non-Hodgkin Lymphoma (NHL). An epidemiological study by Ward *et al*. (2006) conducted in Iowa examined the incidence of NHL among users of both public and private water supplies. In total, 181 cases of NHL and 142 control subjects were included in the study. To determine drinking water nitrate concentration, historical water quality data from 1960-2000 were used for public water supply systems. Private well nitrate concentrations were determined through a combination of sampling the water quality at the time of the study and historical water quality data from the public water system. However, it was discovered that the majority of cases and control populations used water sources with very low nitrate

concentrations; only 11 of the 323 participants studied exceeded the regulatory limit of 10 mg/L NO_3-N (Ward *et al.*, 2006). No significant risk for NHL was associated with elevated drinking water nitrate concentrations below 3 mg/L NO_3-N; further conclusions on the association between drinking water nitrate and NHL were not possible due to the small range of nitrate concentrations in this study (Ward *et al*., 2006).

Another drinking water nitrate and NHL incidence study by Ward *et al*. took place in Nebraska in 1986 (Ward *et al*., 1996). This case-control study examined 156 cases of NHL in white men and women 21 years of age or older who were diagnosed between 1983-1986. The cases were then matched to 527 control cases based on race, gender, vital status, and age; living cases were matched with living control cases, while deceased cases were matched with deceased control cases with the same year of death. The authors interviewed cases and control cases to determine the source and intake of drinking water, and calculated nitrate exposure from 1947-1979 based on historical water quality data. An additional set of 51 cases and 150 control cases who used a private well were interviewed in 1986 and a sample of the water was analyzed for nitrate content. For users of public water sources, long-term consumption was positively associated with increased risk of NHL when nitrate concentrations were at or above 4 mg/L NO_3-N. This association was not present for dietary nitrate, which did not affect risk of NHL in this study. For private well users, no significant increase in NHL risk was observed after adjustment for pesticide use and familial history of cancer. Twenty-one percent of community wells exceeded the MCL of 10 mg/L NO_3-N for 1 year or more; twenty percent of private wells also exceeded this standard. The average nitrate

concentrations for both types of water sources were far below 10 mg/L NO_3-N (Ward *et al*., 1996).

A limited study of NHL incidence and drinking water nitrate concentration was conducted in Sardinia, Italy (Cocco *et al*., 2003). Using data for 153 "communes" (Italian administrative units) with a total population of 703 000, NHL incidence from 1974-1993 was compared with drinking water nitrate concentration as measured in 1993. "Limited evidence" (Cocco *et al*., 2003) was found to support an increased NHL risk with increased nitrate concentration in men only; however, the drinking water nitrate concentrations in the study areas were very low (<27 mg/L NO_3^-), preventing examination of populations exposed to high-nitrate drinking water. Also, because no provision was made for nitrate concentration change over time, the results of the study may be inaccurate.

Another study of drinking water nitrate and NHL incidence was performed in Minnesota, a state which heavily utilizes nitrogen fertilizers (Freedman *et al*., 2000). Only white males, aged 30 years or older, who utilized public or bottled water for their primary drinking water source were considered; users of private wells were not included in this study. Using local drinking water quality information from 1947-1975, and an assumed nitrate concentration of 0 mg/L for bottled water, 73 cases of NHL were compared to 147 healthy control subjects. As with other studies, very low concentrations of nitrate which did not exceed the MCL were found in all of the water sources (maximum NO_3-N concentration: 7.2 mg/L). Based on this limited information, the authors concluded that no association was found between nitrate concentrations below

2.4 mg/L NO_3-N in public water supplies and NHL risk in this population (Freedman *et al*., 2000). However, conclusions about NHL risk at drinking water nitrate concentrations above the MCL cannot be drawn from this study due to the low nitrate concentrations observed.

The relationship between NHL incidence and drinking water nitrate concentration was also examined in the United Kingdom by Law *et al*. (1999). All cases of NHL diagnosed in individuals 0-79 years old between 1984-1993 were compared against nitrate concentrations from 1990-1995 in the Water Supply Zone serving each case. Earlier nitrate measurements were not available. After correction for age and population density, no association was found between drinking water nitrate concentration and NHL incidence (Law *et al*., 1999). This study, like many others in the field, only examined cases utilizing public water supplies, and thus is unable to characterize risk associated with private drinking water wells.

Based on this information, it appears that there is epidemiological evidence both to support and refute the role of drinking water nitrate exposure in the development of NHL. Due to the limitations of these studies, especially the size of the population tested and the widely varying nitrate concentrations of private water supplies, finding a significant result is difficult. Further studies, with larger populations and well-defined drinking water nitrate concentrations, are required before the relationship between nitrate and NHL may be determined.

2.5.2.2 Cancers of the gastrointestinal tract

Drinking water nitrate concentration has also been investigated for an association with increased gastrointestinal cancer risk, often with inconsistent results. A death certificate-based case-control study was conducted in Taiwan to examine any relationship between drinking water nitrate concentration and gastric cancer mortality (Yang *et al*., 1998). All cases of gastric cancer death between 1987-1991 were matched with control cases who had died at the same time from causes unrelated to cancer, gastrointestinal, cardiovascular, or cerebrovascular diseases. Cases and control populations were matched for sex, birth year, death year, residence, and place of death; water quality data collected in 1990 was then matched to cases and control populations based on place of residence. Only public water sources were considered in this study, and mean nitrate concentrations were very low (control: 0.44 mg/L NO_3-N, case: 0.45 mg/L NO_3-N). Despite low nitrate concentrations in the study area, a significant ($p<0.05$) positive association was found between drinking water nitrate concentration and gastric cancer mortality in this study (Yang *et al*., 1998).

Conversely, a study examining the incidence of gastric and esophageal cancer in Yorkshire, United Kingdom, did not find an association with public drinking water nitrate concentration (Barrett *et al*., 1998). All cases of gastric and esophageal cancer in persons older than 15 years diagnosed between 1975-1994 were examined and matched to an appropriate Water Supply Zone (WSZ) based on residency. Historical data for water quality in each WSZ was available from 1990-1995; all nitrate concentrations were below the current regulatory limit (maximum concentration: 40 mg/L NO_3^-). After analysis of 15544 cases of gastric cancer and 5399 cases of esophageal cancer, no

association was found between either cancer and drinking water nitrate concentration (Barrett *et al*., 1998). (Note: this study also examined the incidence of brain cancer, which will be discussed in Section 2.5.2.3.)

Drinking water nitrate concentrations have also been investigated for a possible association with colorectal cancers. De Roos *et al*. (2003) performed a case-control study in Iowa which examined 376 colon cancer cases, 338 rectal cancer cases, and 1244 control subjects who were matched by age and sex. The drinking water nitrate intake of cases and control populations was determined using historical data from the local public water system, based on residency. Intake was calculated from 1960 until time of diagnosis or until 1987 for control subjects. Only subjects who used public water supplies were included in this study. Food intake was also considered and determined by questionnaire. Upon analysis, a "negligible" association of colon and rectal cancer was found with drinking water nitrate concentration. Slightly elevated risk was noted for individuals who were exposed to drinking water nitrate concentrations greater than 5 mg/L NO_3-N for more than 10 years. This finding was not considered highly significant. However, after adjusting for several dietary and health factors, it was discovered that individuals in susceptible subpopulations may be at increased risk of colon and rectal cancer in high-nitrate drinking water areas. Increased susceptibility groups in this study include individuals with low vitamin C and high meat intake, as well as those with conditions causing bowel inflammation (De Roos *et al*, 2003).

As with other adverse health effects, the relationship between drinking water nitrate and gastrointestinal tract cancer is unclear. Few studies have been performed, and many of

these are hampered by small sample size or inconsistent drinking water nitrate concentrations. There is some evidence to suggest that a relationship between these two factors does exist, but further, more comprehensive studies are required to fully explain the nature of this relationship.

2.5.2.3 Cancers of the brain or Central Nervous System

Drinking water nitrate concentration has been investigated for associations with several cancers of the brain and central nervous system (CNS). As previously noted, a study which examined the incidence of gastric and esophageal cancer in the United Kingdom also examined the incidence of brain cancer (Barrett *et al*., 1998). A summary of the study may be found in the above section (2.5.2.2). Along with the cases of gastric and esophageal cancer, 3441 cases of brain cancer were also examined and matched to the Water Supply Zone of their residence. A significant ($p<0.01$) increase of brain and CNS cancer risk was observed with increasing nitrate concentration in this study (Barrett *et al*., 1998). All drinking water nitrate concentrations in this study were below the current regulatory limit of 45 mg/L NO_3^- (maximum observed concentration: 40.1 mg/L NO_3^-). The observation of increased cancer risk at permissible nitrate concentrations requires further study to better characterize the risk, and, if necessary, revise the drinking water standard.

However, a study of drinking water and diet nitrate intake in Nebraska did not show an increased risk of glioma in white men and women over 21 years of age (Ward *et al*., 2005). The study examined 130 cases of glioma diagnosed between 1988-1993 and 319 control subjects. For subjects served by public water systems, historical water quality

data from 1964 onward was used to calculate their drinking water nitrate exposure; a food frequency questionnaire was also used to determine nitrate and nitrite intake. For subjects served by private wells (63 cases, 72 controls), historical data was unavailable; however, researchers obtained water samples at the time of study. The length of time each subject had used a private water well was also recorded. Most public and private water sources were within the regulatory limit for nitrate concentration; however, a few violations were noted for public (12 mg/L NO_3-N) and private (67 mg/L NO_3-N) sources. Despite this, no association was found between public drinking water nitrate concentrations in Nebraska and glioma risk. Conclusions about private well nitrate concentrations and glioma risk were not possible due to the lack of historical data. However, use of a private well was associated with a significant increase in glioma risk for men: risk increased by 2.5-fold with 30 or more years of private well use, and 3.1-fold with 15 or more years of private well use after 1964 (Ward *et al*., 2005). More research is required to determine whether this increased risk is due to nitrate or another factor.

2.5.2.4 Cancers of the pancreas

Several epidemiological studies conducted in the Midwestern United States have examined the relationship between drinking water nitrate and pancreatic cancer risk. One study examined nitrate and nitrite intake in both drinking water and diet in 128 white Iowans aged 40-85 diagnosed with pancreatic cancer between 1985-1987 (Coss *et al*., 2004). These subjects were age, sex, and race-matched to 1244 control subjects; all subjects obtained drinking water from public sources and completed food frequency questionnaires to quantify nitrate and nitrite intake. The average nitrate concentration of

each public water source since 1960 was used to determine average drinking water nitrate intake per year. After analysis, no association was found between drinking water nitrate concentration and pancreatic cancer risk; however, most public drinking water nitrate concentrations were well below the regulatory limit. A "borderline" significant 50% increase in pancreatic cancer risk was associated with 1-2 years of drinking water nitrate concentrations equal or greater than 10 mg/L NO_3-N, but this risk did not persist beyond 2 years (Coss *et al*., 2004). An increased dietary nitrite consumption, particularly of nitrite-rich meat products, was associated with an elevated risk of pancreatic cancer for both sexes.

2.5.2.5 Cancers of the urinary system

Drinking water nitrate has also been suspected to play a role in the development of urological cancer. A case-control study in Iowa was performed by Ward *et al*. (2003), which examined if an association existed between nitrate concentrations in public drinking water and the incidence of bladder cancer. This study examined 808 subjects aged 40-85 who were diagnosed with bladder cancer between 1986-1989, as well as 1259 healthy control subjects. Nitrate intake from public-source drinking water was estimated from 1960 through the use of historical water quality data; users of private wells were excluded from the study. Diet was also examined through a food frequency questionnaire. Upon examination of the data, no associations were found between public drinking water nitrate concentration and bladder cancer incidence. In the subset of subjects who were exposed to nitrate concentrations greater than 10 mg/L NO_3-N, no association was found between years of exposure and bladder cancer incidence. However, very few subjects were exposed to nitrate concentrations above 10 mg/L NO_3-

N, which may have masked effects caused by this level of exposure. The authors conclude that there is no increased risk of bladder cancer with average increasing nitrate concentration in Iowa public drinking water (Ward *et al*., 2003).

The relationship between drinking water nitrate concentration and bladder cancer has also been examined in the Netherlands. Zeegers *et al*. (2006) performed a cohort study which examined men and women who were 55-69 years old in 1986, and followed them for a period of 9.3 years. Nitrate intake from both public drinking water and food was examined and quantified through local water quality data and a food-frequency questionnaire. In total, 871 subjects diagnosed with bladder cancer and 4359 "subcohort members" were studied. No significant association for baseline dietary or drinking water intake, or total nitrate exposure, was found for the incidence of bladder cancer in this study. Despite this, the authors state that an association between bladder cancer and nitrate exposure is "biologically plausible", but that such an association was not found in this population (Zeegers *et al*., 2006).

A unique investigation into drinking water nitrate concentration and the incidence of urological malignancies was conducted in Germany by Volkmer *et al*. (2005). This community-based cohort study examined a small city (~70 000 inhabitants) which was serviced by two different public waterworks for a period of 28 years. One waterworks, which served 57253 residents, had a nitrate concentration of 60 mg/L NO_3^-; the other, which served 10037 residents, had a nitrate concentration of 10 mg/L NO_3^-. The authors examined the incidence of several urological cancers in residents receiving higher (60 mg/L NO_3^-) or lower (10 mg/L NO_3^-) nitrate in drinking water. Nitrate concentration did

not appear to have an effect on the incidence of prostate cancer or renal cell carcinoma in this group, and an inverse correlation with testicular tumours was found. However, a direct correlation with urothelial cancer (cancers of the renal pelvis, ureter, urinary bladder, and urethra) was found in both genders. This study is unique because it compares individuals who inhabit the same community but are exposed to different well-documented nitrate concentrations in their drinking water. An important weakness of this paper is that the authors did not appear to be aware of the different systems of measurement for nitrate concentrations (NO_3^- vs. NO_3-N); however, this does not appear to have affected the validity of their results.

2.5.2.6 Other cancers

A major study of the effects of drinking water nitrate concentration and cancer risk in older women was conducted in Iowa by Weyer *et al*. (2001). This study examined 16541 randomly-selected women aged 55-69 in 1986 and obtained information on food intake (via a food frequency questionnaire) and drinking water source. Public drinking water nitrate concentration was determined from historical community water quality data, and the average nitrate intake for each subject was estimated for the period 1955-1988. While no historical water quality data was available for subjects utilizing private wells, the use of a private well was included as a category in the analysis. The cohort was then monitored from 1986-1998 for the development of cancer. Many different cancers were monitored in this study, including those of the GI tract, bladder, kidney, lung/bronchus, breast, ovary, uterus, pancreas, and skin. Cases of hematopoietic cancer and Non-Hodgkin Lymphoma were also monitored. After adjustment for both risk and protective factors, little association between nitrate concentration and cancer risk at all

sites combined was observed. However, when each cancer was considered individually, positive associations were observed for both bladder and ovarian cancer. The risk of ovarian cancer was increased for women who used a private well compared to those who used a municipal water source with less than 0.36 mg/L NO_3-N. Inverse associations were noted for the risk of uterine and rectal cancer; these results were not expected and may be due to chance (Weyer *et al*., 2001). These results indicate a potential role for drinking water nitrate in cancer development; however, more information is needed on nitrate exposure from private wells before this role can be fully elucidated.

Based on available epidemiological data, the role of drinking water nitrate in cancer development is uncertain. Conflicting evidence is available for Non-Hodgkin Lymphoma, gastrointestinal cancer, brain and central nervous system cancer, pancreatic cancer, urinary system cancer, and other cancers. Many of these studies have not examined populations reliant on private drinking water wells. The majority of studies available have found no positive link between cancer incidence and nitrate concentration in public water systems. However, public water supplies are often subject to constant quality monitoring, with prompt remediation of guideline violations. Conversely, private water supplies may not be tested frequently and may contain nitrate concentrations much greater than the regulatory limits. More studies which incorporate private water sources must be performed to gain a complete understanding of the role of drinking water nitrate in cancer incidence, particularly considering the large proportion of rural residents who are reliant on private wells.

2.5.3 Nitrate and diabetes

Nitrate has also been suspected to have a diabetogenic effect on children. Several studies conducted in Europe have attempted to determine if a relationship exists between drinking water nitrate concentration and childhood-onset Type 1 insulin-dependent diabetes mellitus (IDDM). An ecological analysis of 1797 0-16 year old children diagnosed with IDDM between 1978-1994 in Yorkshire, UK examined drinking water nitrate concentrations from 1990-1995 in the water supply zone (WSZ) where each child lived (Parslow *et al.*, 1997). Upon comparison to expected incidences of IDDM for each area, a significantly elevated incidence of IDDM was observed in areas with drinking water nitrate concentrations above 14.85 mg/L NO_3^-. Few areas, however, exceeded the regulatory nitrate limit of 50 mg/L NO_3^-, with only 8% of WSZs ever exceeding this limit between 1990-1995. Despite this discovery, the authors caution that their study should be considered "hypothesis-generating", and that more study on a larger scale is required.

Another ecological study into IDDM and drinking water nitrate was conducted in the Netherlands by van Maanen *et al*. (2000). The incidence of IDDM in children 0-14 years old, diagnosed between 1993-1995, was compared to the drinking water nitrate concentrations from 1991-1995 in the same postal code area where the children resided. A total of 1064 IDDM cases were analyzed, and the results were compared against the expected incidence in each area. Upon analysis, the concentration of drinking water nitrate did not have an effect on the incidence of IDDM in this study. None of the areas studied, however, exceeded the regulatory limit of 50 mg/L NO_3^-, limiting the scope of the study.

An additional study into IDDM and drinking water nitrate concentration in the United Kingdom was performed by Zhao *et al*. (2001). This study was similar to the Parslow *et al*. (1997) study in that it examined the incidence of IDDM in children and compared this to the nitrate concentration of the water supply zone where each child lived. However, this study examined 517 children 0-15 years old, diagnosed with IDDM between 1975-1996 in the Cornwall and Devon areas of England. Water quality data from 1993-1997 was used to determine average drinking water nitrate intake. Socioeconomic status was also considered in this analysis. In contrast to Parslow *et al*. (1997), this study found no significant relationship between drinking water nitrate concentration and IDDM incidence. In one univariate analysis, nitrate appeared to have a borderline protective effect, though the authors doubted the validity of this finding (Zhao *et al*., 2001). The average nitrate concentration of each WSZ was far below the regulatory limit of 50 mg/L NO_3^-, and no WSZ ever exceeded the limit (maximum nitrate concentration: 31.9 mg/L NO_3^-). The authors caution that more studies on this topic, including case-control studies, are required.

The high incidence of IDDM in Finnish children under 14 inspired an ecologic study by Moltchanova *et al*. (2004) to determine if this observation was linked to groundwater nitrate concentration. Groundwater was chosen because it is used as a source of drinking water in Finland and because a recent national hydrogeochemical study had been conducted, providing recent water quality data. A total of 3564 children under 15 years old, diagnosed with IDDM between 1987-1996, were used in this study. A nonsignificant tendency of increasing IDDM risk with increasing groundwater nitrate

was observed. Rurality, which was included as a covariate, did not appear to increase the risk of IDDM. Residents of rural Finland are often served by private wells, so the inclusion of rurality as a covariate provided vital information on a potentially vulnerable population.

Based on available information, it is unclear whether nitrate influences the risk of developing Type 1 insulin-dependent diabetes. Limitations on study size and range of nitrate concentrations have resulted in an incomplete picture of the effects of drinking water nitrate. More research in this area is required before the relationship between drinking water nitrate and Type 1 diabetes is understood. The development of large-scale case-control studies, as opposed to the ecologic studies currently available, may also serve to better investigate this relationship.

2.5.4 Nitrate and thyroid disorders

Drinking water nitrate exposure is also suspected to cause or contribute to a number of thyroid disorders. However, epidemiological studies have provided inconsistent results, and the role of nitrate in these conditions is unknown. Two European studies have indicated a possible connection between drinking water nitrate concentrations and the incidence of thyroid disorders or subclinical effects.

The first study, conducted by Tajtáková *et al*. (2006) examined Slovakian children aged 10-13 years old for subclinical thyroid disorders, and compared the findings to the relative nitrate concentrations in each child's residential area. Three groups of children were studied: 324 children from a high-nitrate agricultural area (51-274 mg/L NO_3^-),

168 children from a low-nitrate agricultural area (<2 mg/L NO_3^-), and 596 children from a nearby city with a low-nitrate drinking water supply. These children were examined to determine thyroid volume and echogenicity, signs of thyroid sufficiency. Children with pre-existing thyroid conditions were excluded from the study. Blood samples, used to quantify concentrations of thyroid hormones and anti-thyroid antibodies, were obtained from 315 high-nitrate and 109 low-nitrate children. The study found that thyroid volume was significantly larger in children from high-nitrate areas compared to low-nitrate children. Hypoecogenicity, a sign of damaged thyroid tissue, was found significantly more often in the high nitrate group compared to pooled data from the low-nitrate areas. Also, subclinical hypothyroidism, determined by blood thyrotropin levels, and thyroperoxidase antibodies, indicative of an autoimmune response, were found only in the high-nitrate group. The authors note, however, that these findings were very rare and may have occurred only in the high-nitrate group by chance. Based on these findings, the authors conclude that exposure to high drinking water nitrate in this group may increase thyroid volume and signs of subclinical hypothyroidism (Tajtáková *et al*., 2006).

Another study, conducted in the Netherlands by van Maanen *et al*. (1994), examined healthy adult female volunteers who used public or private drinking water sources. The subjects were grouped on the basis of their nitrate exposure: groups A and B both utilized public drinking water sources, while groups C and D used private water wells. Group A consisted of 24 women with the lowest public drinking water nitrate concentrations (0.02 mg/L NO_3^-), while Group B included 27 women with medium public drinking water nitrate concentrations (17.5 mg/L NO_3^-). Group C consisted of 7

women who used private drinking water wells with a nitrate concentration less than 50 mg/L NO_3^-, and Group D consisted of 12 women with the highest nitrate exposure, using private wells with a nitrate concentration over 50 mg/L NO_3^-. These subjects completed a food frequency questionnaire, gave samples of saliva and blood, underwent 24 hour urine collection, and were examined with ultrasonography and palpitation of the thyroid. A significant increase in thyroid volume (thyroid hypertrophy) was observed in Group D compared to Groups A, B, and C. Women in Group D also showed signs of hyperthyroidism, including significantly decreased thyroid stimulating hormone (TSH) levels and significantly increased thyroxine levels, compared to women in the other groups. Thus, a significant difference in thyroid volume and function was observed in women consuming water with a nitrate concentration greater than 50 mg/L NO_3^- compared to those exposed to nitrate below the regulatory limit. This study is unique in that the authors performed their own analyses of the subjects' drinking water; samples of both public and private sources were taken at home visits. The inclusion of subjects reliant on private drinking water wells was also unique, as this population is often excluded from drinking water studies.

The two studies described above provide evidence supporting a role for drinking water nitrate in the development of thyroid disorders. However, the small sample size of each study may have produced biased results. Although these studies have examined the effects of private well water exposure, more samples with better-defined nitrate concentrations would provide a greater understanding of the relationship with the incidence of thyroid disorders. The availability of historical water quality data might

also permit a better understanding of critical periods or the time course of thyroid disorder development.

2.5.5 Nitrate and teratogenic or reproductive effects

Nitrate exposure has long been suspected to contribute to adverse teratogenic and reproductive effects in humans. Many studies examining the role of nitrate exposure and teratogenicity or adverse reproductive effects have been conducted in Canada; these studies are described in section 2.6.2. The following is a selection of relevant teratogenic or reproductive studies conducted outside Canada.

A study conducted in the United States by Brender *et al*. (2004) examined the role of dietary nitrate and nitrite, nitrosatable drugs, and fetal neural tube defects (NTDs) in Mexican-American women living along the Texas-Mexico border. A subsection of the study group, consisting of 43 case women and 67 control women, underwent analysis of their drinking water to determine nitrate content. The authors of this study conducted their own nitrate analyses using samples collected during home visits. The drinking water nitrate concentration for this group ranged from 0-28 mg/L NO_3^-, with no samples exceeding the regulatory limit of 45 mg/L NO_3^-. However, women with a drinking water nitrate concentration exceeding 3.5 mg/L NO_3^- were 1.9 times more likely to have a child with an NTD than a woman with a lower nitrate concentration. This effect was enhanced if women also took nitrosatable drugs during the pregnancy. An important note about this study is that it did not take the actual amount of water consumed into account, nor did it consider other water sources outside the home. The authors caution

that the effects observed may be due to bias from a small sample size, rather than an effect of nitrate itself (Brender *et al*., 2004).

Nitrate was also investigated for its possible teratogenic effects in rural south Australia by Dorsch *et al*. (1984). This case-control study was conducted in a region of Australia with a significantly increased incidence of NTDs. The study population consisted of 218 case-control pairs who had given birth between 1951-1979 and were matched by hospital, maternal age, parity, and date of birth. The drinking water source for each woman was determined to be either rainwater, groundwater, or water from a nearby lake. The groundwater in this region was highly polluted with nitrate, causing several public wells to have a nitrate concentration well above the regulatory limit of 45 mg/L NO_3^-. Using government water quality data, the nitrate content of each subject's water source (including public wells) was determined. Other drinking water contaminants were discussed in the article, but it does not appear that each subject's water was tested for other contaminants. Women who used a private well for drinking water were 4.1 times more likely to have an infant with a congenital deformity compared to those who used rainwater as their primary source; those who used public water supplies had a relative risk of only 1.4. When water sources were analyzed by nitrate content, women who used sources with nitrate concentrations above 5 mg/L NO_3^- had a greater risk of teratogenesis compared to those who used lower-nitrate drinking water sources. This risk only extended to the risk of NTDs and oral clefts; significant associations were not found with other congenital defects. Although the authors hypothesize that an unidentified substance that occurs concurrently with nitrate may be responsible for the defects, they note that this study is not designed to test that hypothesis (Dorsch *et al*,

1984). While this study is widely cited by researchers examining the teratogenic potential of nitrate, it has also been criticized (Fan & Steinberg, 1996) for its methods and conclusions.

An investigation into a potential role for drinking water nitrate in reproductive failure was spurred after several reports of spontaneous abortions in four Indiana women living in close proximity (CDC, 1996). A cluster of four women who had suffered at least one spontaneous abortion was identified. One woman had experienced two spontaneous abortions after moving into a house with a nitrate-contaminated private water well. Upon analysis of the well water, nitrate was the only contaminant present at elevated levels (>10 mg/L NO_3-N). All wells used by the women were suspected to be contaminated either by a nearby hog-confinement facility or a septic tank. After switching to low-nitrate sources of drinking water, each woman gave birth to one or more live, full-term children. While the editors caution that this observation may be coincidental, they state that this case, combined with research indicating nitrate may cause spontaneous abortions in cattle and laboratory animals, highlights the need for further investigation of nitrate in reproductive failure (CDC, 1996).

As with other suspected toxic effects of nitrate, involvement in teratogenic or adverse reproductive outcomes is controversial. Evidence both supporting and refuting a role of drinking water nitrate in these conditions is available. Some reports, such as the spontaneous abortion report described above (MMWR, 1996), are more anecdotal and may have occurred by chance. Small sample size may also have created bias in several of these studies. Due to the severity of these outcomes, and several findings which have

indicated increased risk at nitrate concentrations below current regulatory limits, more research is required to fully comprehend the role of drinking water nitrate in these conditions.

2.6 Nitrates in Canada

2.6.1 Nitrate concentrations in Canada

Nitrate concentrations in Canadian drinking water, while not as potentially hazardous as those of developing countries, may still pose a threat to human health. Generally, most nitrate concentrations in Canadian public water supplies are below 5 mg/L NO_3^-, far below the MAC of 45 mg/L NO_3^- (Health Canada, 1992). However, private wells, which are often fed from groundwater sources, may contain much higher concentrations. Excessive groundwater nitrate concentrations have been reported in Ontario (467 mg/L NO_3^-) and Manitoba (1063 mg/L NO_3^-) (Health Canada, 1992). These concentrations may pose a risk to human health.

Intensive agricultural practices in Canada may increase the amount of nitrate present in groundwater and drinking water. A study performed in Portneuf county, Québec found that significant groundwater and private well nitrate contamination was found in areas undergoing intensive potato cultivation. This contamination was enhanced by the sandy quality of the soil, thus allowing greater nitrate mobility, and did not appear to be affected by livestock, other agriculture, or sanitary sewage systems in the area (Levallois *et al*., 1998). In Prince Edward Island, significantly higher nitrate concentrations were found in private water wells in proximity to potato, grain, and hay fields (Benson *et al*., 2006). Anthropogenic contributions, including agriculture and sewage systems, may be

responsible for significant increases in nitrate and nitrite observed in the Elbow River west of Calgary. This source provides drinking water for half of the city of Calgary (Sosiak & Dixon, 2006). A survey of farm drinking water wells in Ontario performed between 1991 and 1992 showed that 45% of the sampled sites exceeded the MAC for nitrate in both the summer and the winter (Rudolph *et al.*, 1998). Many of the farms whose wells exceeded the MAC engaged in over-fertilization of their cropland, applying both animal manure and inorganic fertilizer. This created an excess of nitrogen in the soil, and may have contributed to the contamination of the groundwater through nitrate leaching (Rudolph *et al.*, 1998). A similar situation was found near Lethbridge, Alberta, where 13 of 16 piezometers placed in agricultural fields receiving both inorganic fertilizer and manure showed nitrate concentrations exceeding the MAC (maximum nitrate concentration: 52 mg/L NO_3-N) (Rodvang *et al*., 2004).

An investigation into the sources of total daily nitrate intake in four areas of rural Québec examined the contributions of food and drinking water for 187 rural residents reliant on private wells for their water supply (Levallois *et al*., 2000). While food was the primary source of nitrate exposure, consumption of “moderately” nitrate-contaminated water (7 mg/L NO_3-N) provided 30% of total nitrate intake. The authors conclude that nitrate-contaminated drinking water can “significantly” contribute to total body burden (Levallois *et al*., 2000). This suggests that the consumption of contaminated drinking water may be a significant source of nitrate intake in areas of Canada where even moderate nitrate contamination occurs.

While Canadian drinking water sources are generally safe, private wells may pose an additional hazard. Private wells may not be inspected regularly, so nitrate contamination may go unnoticed. Intense agricultural production in some regions of Canada appears to have locally increased drinking water nitrate concentrations. Often, poor land management strategies (e.g. overfertilizing with inorganic and organic fertilizers) significantly contribute to local nitrate contamination. Drinking this contaminated water can significantly contribute to daily nitrate intake and may increase the body burden of nitrate. Careful and accessible management strategies, as well as regular private well testing, is required to protect the health of Canadians reliant on private drinking water wells.

2.6.2 Nitrate and epidemiological studies performed in Canada

Several Canadian epidemiological studies have been performed to determine if there is an association between nitrate exposure and adverse health outcomes in Canada. Several of these studies have examined the relationship between maternal drinking water intake and subsequent neonatal pathology, such as neural tube defects, CNS defects, intrauterine growth restriction and prematurity, and brain tumours. Other studies have examined the incidence of various cancers and drinking water nitrate intake in adult populations. Due to the prevalence of agricultural activity in Canada, and the large proportion of the population which resides in rural areas, epidemiological studies of nitrate exposure are vital to understanding the potential adverse health outcomes which may occur.

The teratological potential of maternal nitrate exposure has been investigated in several provinces. One such study examined the incidence of neonatal neural tube defects, including conditions such as aencephaly, spina bifida cystica, and encephalocele, in Newfoundland from 1976-1980 (Frecker & Frasier, 1987). The investigators examined records of infant births and stillbirths to determine deaths attributed to neural tube defects (NTDs), and discovered 267 applicable cases. These cases were then organized by geographical location, represented by census district, and compared with nitrate concentration data for nearby lakes. Water quality data for nearby lakes were used due to the absence of drinking water quality data for this period. After analysis, it was determined that no positive correlation was observed between lake water nitrate concentration and NTD incidence (Frecker & Frasier, 1987). However, it is important to note that the composition of the lake water does not necessarily correspond with the characteristics of the local drinking water; thus, an accurate determination of maternal nitrate drinking water exposure cannot be made.

Another investigation into nitrate teratology was conducted in New Brunswick by Arbuckle *et al*. (1988). This study examined 130 cases of congenital CNS malformations which occurred in six designated “study counties” between 1973 and 1983. Three counties were considered to have a high prevalence of congenital CNS defects (“high risk counties”); three with a low CNS defect prevalence were also included (“low risk counties”). Each identified case was matched to two control infants with similar birth dates and maternal residence location. Questionnaires were administered to identify factors, such as parity, parents’ birthplace, ethnicity, socioeconomic status, and drinking water source (municipal, private well, spring, or

bottled). Several samples of drinking water were taken and tested for nitrate, chloride, fluoride, and sulphur.

Upon analysis of the data, it was revealed that the source of drinking water appeared to modify the relationship between nitrate concentration and the risk of CNS defects. A positive trend was observed between drinking water nitrate concentration and adjusted risk odds ratio of CNS defects when the maternal source of drinking water was a private well. Although this trend was not statistically significant, the authors describe it as "moderate" (Arbuckle *et al*., 1988). An inverse relationship between nitrate concentration and CNS defect risk was observed when the maternal drinking water source was a municipal water supply or a natural spring. The relationship was not statistically significant, but presents an interesting comparison to results obtained for well water. Generally, elevated drinking water nitrate concentrations were identified in "high-risk" counties as opposed to "low-risk" counties. In the lower-risk regions, 87.5% of the water samples contained 1.65 ppm (1.65 mg/L NO_3^-) of nitrate or less; however, in the higher-risk regions, 87.5% of the samples contained 26ppm (26 mg/L NO_3^-) of nitrate or less. This suggests that there is a greater prevalence of drinking water nitrate in the "high-risk" counties; it should be noted, however, that of the 304 households undergoing water analysis, only 5 households (3 control and 2 case) exceeded the Health Canada MAC for nitrate (45 mg/L NO_3^- or 45 ppm) (Arbuckle *et al*., 1988).

Based on these findings, the investigators conclude that nitrate cannot be viewed as a "strong risk factor" for congenital CNS defects in the areas studied (Arbuckle *et al*., 1988). This finding contradicts a previous study, performed in Australia, which found

that maternal drinking water nitrate concentration was a significant risk factor for congenital CNS and musculoskeletal system defects (Dorsch *et al*., 1984). The authors of the present study proposed several explanations for this disparity, including differing water chemistry or susceptibility factors between the two areas, generally lower concentrations of drinking water nitrate in New Brunswick as opposed to Australia, and insufficient consideration of potential confounding factors in the Dorsch *et al*. (1984) study. More research is needed to elucidate why nitrate appears to be a risk factor for congenital CNS defect when consumed in well water, but not municipal or spring water.

Nitrate has also been investigated for a potential link to prematurity and intrauterine growth restriction (IUGR) in Prince Edward Island infants (Bukowski *et al*., 2001). The investigators identified 210 cases of IUGR and 336 cases of premature birth born between 1991 and 1994, and compared these cases to 4098 healthy control infants born in the same time period. This data was then compared with groundwater nitrate concentration; the authors created a nitrate level exposure map based on areas with similar water sources and matched the case and control infants to areas based on the mother's postal code. Groundwater nitrate concentration was examined because it is the source for both municipal and private drinking water wells in Prince Edward Island. Historically, agriculture has increased the concentration of groundwater nitrate on the Island; approximately 7% of wells in agriculture-intensive areas exceed Health Canada's MAC of 45 mg/L NO_3^- (Bukowski *et al*., 2001). Areas with high and low agricultural activity were included in this study.

In order to appropriately identify confounding factors, the investigators analyzed interactions with maternal age, pre-pregnancy weight and height, smoking status, alcohol consumption, presence of chronic disease (e.g. diabetes, hypertension, heart disease), parity, previous abortion history, problems with previous pregnancies, maternal stress, maternal nutrition status, pregnancy-induced or related disease status, and febrile episodes. Incidences of multiple births (twins, triplets, etc.) were excluded from this study because lower birth weight is common in these situations. Regional socioeconomic data were included in the analysis.

After analysis, both IUGR and prematurity were shown to have a significant positive dose-response relationship with groundwater nitrate concentration (Bukowski *et al*., 2001). Increased IUGR risk was not observed in the highest range of nitrate concentrations; however, there were only three cases included in this category so the statistical power is very low. Based on these results, it appears that groundwater nitrate concentration may significantly correlate with the incidence of IUGR and prematurity in Prince Edward Island. Since groundwater nitrate concentrations were generalized over a geographical area, variations in individual nitrate intake were not considered. The use of regional socioeconomic data, as opposed to individual information, may have also introduced bias. Due to these complicating factors, further study is required to confirm these observations. The observed relationship between nitrate and IUGR/prematurity may also be indicative of a different causative factor, such as factors related to agricultural contamination of water supplies (e.g. pesticides) (Bukowski *et al*., 2001).

Another study, conducted jointly in Canada and the United States, examined the effect of maternal diet on the risk of development of astrocytic glioma (a common childhood brain tumour) in their children (Bunin *et al*., 1994). 155 cases of astrocytic glioma were identified and reviewed by a pathologist to ensure they were appropriate for the current study. An equal number of control children were identified and matched to each case by geographical location (determined by telephone number), age, and race (defined as Black or non-Black). A telephone interview of both case and control mothers identified factors such as mother's occupation, income, education level, smoking status, family history of cancer, affected child's medical history, and "household, personal, and medical exposures" during gestation (Bunin *et al*., 1994). Because N-nitroso compounds and related substances, including nitrate, were hypothesized to increase risk of astrocytic glioma, a detailed questionnaire on maternal diet, including drinking water, was administered. The frequency of consumption of fruits, vegetables, cured meats, alcohol, caffeine, artificial sweeteners, and raw or rare meats was recorded in order to determine maternal exposure to N-nitroso compounds and their precursors.

After analysis, a positive trend was observed for the consumption of cured meats during pregnancy and the risk of astrocytic glioma in the child (Bunin *et al*., 1994). Cured meats often contain nitrosamines, nitrite, and nitrate; however, subsequent analyses of these compounds separately did not show a strong association with astrocytic glioma development. Analysis of drinking water was restricted to determination of the source: municipal supply, private well, or bottled water. There was no significant difference in water source between case and control mothers. The authors conclude that, based on this study, N-nitroso compounds consumed during pregnancy may increase the risk of

astrocytic glioma development in children. Maternal drinking water consumption from private wells does not appear to increase the risk of astrocytic glioma in their children (Bunin *et al*., 1994). However, this finding must be interpreted with caution; a wide range of water quality parameters may be found in private wells, so determination of the risk associated with well water consumption must be coupled with water quality data. More research is required before the relationship between drinking water nitrate consumption and astrocytic glioma is fully elucidated.

Epidemiological studies have also been conducted in Canada to determine the relationship, if any, between drinking water nitrate and carcinogenesis. An investigation into cancer incidence and drinking water contamination with nitrate and atrazine was conducted in Ontario, using age-standardized cancer data from 1987-1991 (Van Leeuwen *et al*., 1999). The study examined 40 geographical "ecodistricts" (EDs) in Ontario to gain information about relevant agricultural practices, such as land use and pesticide, fertilizer, or manure application. Drinking water concentrations of nitrate and atrazine were also obtained from previous water quality studies of 1300 municipal and private water supplies; these concentrations were then used to develop a general drinking water contamination measurement for the entire ecodistrict. These data were then compared to the local incidence of cancers thought to be associated with atrazine or nitrate exposure, such as stomach, colon, ovarian, bladder, CNS, and non-Hodgkin's lymphoma. Corrections for potential confounding factors such as alcohol consumption, smoking status, education level, income, occupation, sex, and age were included (Van Leeuwen *et al*., 1999).

After correction for confounding factors, a positive and significant ($p<0.05$) correlation was found with atrazine drinking water concentration and stomach cancer incidence in both males and females; however, a significantly *negative* correlation was found for colon cancer incidence. Nitrate drinking water concentration showed a significantly negative correlation with stomach cancer in both sexes, but was significantly positively associated with colon cancer incidence in females only (Van Leeuwen *et al*., 1999). The other cancer types investigated did not show a significant relationship with either atrazine or nitrate drinking water concentration. The finding that nitrate concentration is not associated with stomach cancer incidence does not agree with some previous research on the subject, which found a positive correlation between nitrate concentration and gastric cancer (Yang *et al*., 1998). However, other researchers have failed to find a correlation between drinking water nitrate concentration and gastric cancer incidence (Barrett *et al*., 1998). More research is required before this relationship may be fully characterized. Environmental nitrate and atrazine combine to form nitroso-atrazine, a compound with synergistic carcinogenic potential (Van Leeuwen *et al*., 1999). Therefore, it is possible that nitroso-atrazine is the causative agent. More study of these hypotheses, as well as the potential link between nitrate consumption and colon cancer in females, is required to explain these findings.

The results of epidemiological studies performed in Canada provide conflicting evidence regarding the toxic effects of nitrate. In some instances, nitrate consumption appears to be linked to adverse health outcomes, such as intrauterine growth restriction or prematurity in infants, or colon cancer in women. However, other studies have found no significant association between nitrate consumption and neural tube defects or

astrocytic glioma development in children. A negative correlation between nitrate consumption and stomach cancer in both sexes was found in one study. Some interesting results were found, such as the positive association between nitrate consumption in well water (but not municipal or spring water) and congenital CNS defects in children. However, some caution must be used when interpreting the significance of these results. It is very difficult to correct for all potential confounding factors or exposures in such studies, and assumptions must often be made about levels of exposure or behaviours. Several of the studies reviewed here rely on indirect measurements of nitrate consumption, which may have introduced error. More research is required before a definite explanation for the role of drinking water nitrate in adverse health outcomes can be fully developed, if such an association exists.

2.7 Nitrate concentrations and epidemiological studies performed in Saskatchewan

Several studies exist detailing the concentration, prevalence, and health effects of nitrates in Saskatchewan drinking water. Due to the high proportion of the population living in rural areas of the province (approximately 50%), the local prevalence of agriculture and animal husbandry, and the estimated 66 000 water wells providing drinking water for the residents (Thompson, 2001), there is increased potential for drinking water contamination with nitrate.

A literature review conducted by Henry (1995) concluded that 7-17% of drinking water wells in Saskatchewan may exceed the MAC for nitrate (45 mg/L NO_3^-). Different sources of nitrate may be responsible for differing degrees of contamination; point sources are thought to be responsible for highly contaminated wells, while soil organic

matter is thought to be the causative factor in less contaminated wells (Henry, 1995). A similar literature review in the same article examining the situation in Alberta, however, concluded that drinking water nitrate standards were exceeded less often than in Saskatchewan. Possible explanations for this difference appear to hinge on the different geology and water chemistry present, particularly the type of aquifers found in Alberta compared to Saskatchewan (Henry, 1995).

More recently, Thompson (2001) used private water well quality data from Saskatchewan Health's Provincial Laboratories to determine the condition of well water in the province. Both water wells and dugouts were examined, using samples that private owners had submitted to the Provincial Laboratory for testing. Analysis revealed that 483 of the 3425 wells (~14%) tested in a 12-month period exceeded the MAC for nitrate, with a maximum nitrate concentration of 957 mg/L NO_3^-. However, 50% of tested wells showed a nitrate concentration of 3 mg/L NO_3^- or less. Very few other studies of nitrate concentrations in rural Canadian water supplies have been performed; however, a study of wells in a rural potato-farming area of Québec known to be contaminated with nitrate found that 13.3% exceeded the MAC (Levallois *et al*., 1998). A study of 1497 domestic and public wells across the United States found that approximately 11% exceeded the regulatory limit for nitrate (Squillace *et al*., 2002). This indicates that the nitrate concentrations in rural wells obtained by Thompson are similar to those in other North American studies. Interestingly, Thompson (2001) found that none of the 150 dugouts tested exceeded the MAC, and generally showed a much lower nitrate concentration than the tested wells. The reason for this finding is unknown. Due to the small number of dugouts tested, however, no definite conclusions could be

drawn from the comparison. A small review conducted by Thompson (2001) in the same article indicates that these concentrations and prevalences are similar to those observed in other rural areas of Canada and the United States. Given the common usage of nitrate-based fertilizers in Saskatchewan, and the move towards intensive livestock operations, Thompson suggests that these may be contributing factors to drinking water nitrate contamination in the province. Based on the prevalence of high-nitrate drinking water in Saskatchewan, Thompson concluded that "further study is clearly warranted" on the chronic effects of nitrate-contaminated drinking water exposure (Thompson, 2001).

One study has highlighted a possible link between nitrate concentration and the incidence of multiple sclerosis (MS) in Saskatchewan (Irvine & Schiefer, 1988; Irvine & Schiefer, 1989). The hamlet of Henribourg, Saskatchewan has been shown to be a "cluster focus" of MS cases; that is, a location where several MS cases developed or a location where several future MS cases resided during childhood. After comparison with similar sites in Saskatchewan that had a zero incidence of MS, the authors concluded that Henribourg had a much greater concentration of nitrate and nitrite in the drinking water wells compared to the control site. This value, which was not quantified in the publication, exceeded both the objective standard and the maximum concentration for domestic consumption for the Canadian drinking water standards at the time. After analysis of other water and soil characteristics, the authors conclude that increased concentration of nitrate and nitrite in drinking water *may* be one of several characteristics of a "MS-predisposing environment" (Irvine & Schiefer, 1988; Irvine & Schiefer, 1989). However, the authors caution that this is an observation only and that conclusions about causation cannot be made from this data (Irvine & Schiefer, 1988;

Irving & Schiefer, 1989). More recent studies have indicated that nitric oxide (and its metabolites, nitrate and nitrite) are elevated in MS lesions, indicating a potential role for these compounds in the disease itself (Smith & Lassmann, 2002). Increased consumption of drinking water nitrate may play a role in this relationship, but the possible mechanisms by which this might occur are presently unknown.

Fatalities and near-fatalities from infant methemoglobinemia have also been reported in Saskatchewan. Robertson and Riddell (1949) describe two cases of nitrate-induced methemoglobinemia which resulted in infant fatalities in the Regina area. Both infants had been fed reconstituted powdered milk formula made with nitrate-contaminated private well water. Samples of the water were later analyzed and found to contain 1219 ppm (1219 mg/L NO_3-N) and 1320 ppm (1320 mg/L NO_3-N) nitrate, levels which far exceeded the acceptable concentration of 10 ppm (10 mg/L NO_3-N) (Robertson & Riddell, 1949). Subsequent inspection of the wells found that they were in “deplorable” condition, with clear indication of fecal contamination from domestic farm animals. The authors also briefly describe ten other confirmed cases of non-fatal infant methemoglobinemia in the same area; all of these cases were caused by consumption of nitrate-contaminated well water with nitrate concentrations over 75 ppm (Robertson & Riddell, 1949). Another such case, thought to be the first reported in Saskatchewan, is presented by Goluboff (1948), where an infant near Saskatoon developed nearly-fatal methemoglobinemia after consuming formula containing 213 ppm (213 mg/L NO_3-N) of nitrate. Upon follow-up, the infant appeared to have made a full recovery. Although they are not recent, these case studies illustrate the potential danger to Saskatchewan infants from nitrate-contaminated drinking water.

The province of Saskatchewan is uniquely susceptible to nitrate contamination and its associated adverse effects due to the intensity of agricultural production and the large rural population reliant on private drinking water wells. Excessive drinking water nitrate concentrations have been observed by several researchers. The prevalence of nitrate contamination in Saskatchewan appears to be similar to that of other highly agriculturalized areas in Canada and the United States. Adverse effects associated with nitrate contamination, such as methemoglobinemia, have occurred in Saskatchewan, and recent research indicates that the nitrate contamination may play a role in the development of multiple sclerosis in Saskatchewan communities. More research is urgently required to determine the specific risks facing the people of Saskatchewan from drinking water nitrate contamination.

2.8 Potential mechanisms of nitrate toxicity: previous findings

The precise mechanism of nitrate toxicity has generated intense scientific debate for several decades. Due to the complex toxicokinetics of ingested nitrate, it is difficult to definitively determine the mechanism of nitrate toxicity. Both the toxicokinetics/pharmacokinetics of nitrate, and several hypotheses regarding the molecular mechanism of nitrate toxicity will be presented here.

2.8.1 Pharmacokinetics/toxicokinetics of nitrate

When nitrate is ingested, little absorption occurs in the stomach (Walker, 1996); almost all nitrate is readily and rapidly absorbed in the small intestine (Bartholomew & Hill, 1984). Nitrate is carried in the plasma and rapidly distributed throughout the body, quickly reaching equilibrium in body tissues (Walker, 1996). Approximately 25% of a

nitrate dose is actively secreted into the saliva by the same ion transporter as iodide, perchlorate, and thiocyanate (Tannenbaum *et al*., 1976; Spielgelhalder *et al*., 1976). Oral flora, particularly those located at the base of the tongue, reduce approximately 20% of this secreted nitrate into nitrite (Walker, 1996). This process results in oral reduction of approximately 5% of a total dose of nitrate into nitrite in the oral cavity (Spiegelhalder *et al*., 1976; Walker, 1996). Oral reduction of secreted nitrate to nitrite is thought to be the most important nitrite source for humans (Walker, 1996); however, large variations in oral nitrate reduction exist (Tannenbaum *et al*., 1976). The newly-formed salivary nitrite is then swallowed and reabsorbed (Spiegelhalder *et al*., 1976). Nitrate may also be reduced to nitrite by microorganisms present in the human gastrointestinal tract (Schultz *et al*., 1985). The healthy human stomach is an unlikely location for microbial nitrate reduction due to its hostile environment (Walker, 1996), but individuals with infections or reduced stomach acidity may be prone to bacterial colonization and potential nitrate reduction (WHO, 2004).

Nitrate which is not reduced is excreted in bodily fluids, such as urine, sweat, and saliva. A negligible portion of nitrate may be excreted in the feces (Bartholomew & Hill, 1984). The major route of nitrate excretion is through the urine; approximately 65-70% of the total dose is excreted over 18-24 hours (Bartholomew & Hill, 1984). Tannenbaum *et al*. (1976) estimate the half-life of nitrate to be 12 hours. Peak nitrate concentrations are reached rapidly in saliva (1 hour), sweat (1 hour), and urine (4-6 hours) (Bartholomew & Hill, 1984). Schultz *et al*. (1985) developed a three-compartment pharmacokinetic model to describe the movement of nitrate in the human body; others have used a one-compartment model (Wagner *et al*., 1983). It is important to note, however, that humans

have the capacity to endogenously synthesize nitrate (Green *et al*., 1981). This may significantly contribute to the body burden and toxicity of nitrate. Nitrate and nitrite are produced as breakdown products of nitric oxide (NO). Because NO production can be stimulated in response to infection, increased concentrations of nitrate and nitrite may occur. This can be particularly dangerous for very young infants, who are very prone to the development of methemoglobinemia (Vermeer & Van Maanen, 2001).

Much concern has been expressed over the potential for nitrate and nitrite to form carcinogenic N-nitroso compounds *in vivo*. Exposure to N-nitroso compounds may cause gastric, esophageal, bladder, colorectal, and nasopharyngeal cancers in humans (Vermeer & Van Maanen, 2001). Based on the available evidence, it does appear possible that ingested nitrate and nitrite may form N-nitroso compounds, particularly in the stomach (Spiegelhalder *et al*., 1976; Vermeer & Van Maanen, 2001). After the reduction of nitrate to nitrite in the oral cavity, the newly-formed nitrite is swallowed and enters the stomach. Due to the favourable environment, nitrite can react with water, stomach acid, and amines or amides present to form nitrosamines and nitrosamides. These compounds are potentially carcinogenic (Vermeer & Van Maanen, 2001). Although some compounds, such as ascorbic acid, may block N-nitroso formation, their effectiveness is debatable. There appear to be additional factors which influence N-nitroso formation which have not been fully described; these may explain the variable effectiveness of such protective compounds.

2.8.2 Previous research examining nitrate toxicity at the molecular level

In order to determine the mechanism of nitrate toxicity, Chow and Hong (2002) examined the effects of dietary Vitamin E and selenium supplementation on the survival of rats exposed to high concentrations of nitrate or nitrite. These authors, and other researchers working independently, noticed greater mortality in rats which were fed an anti-oxidant-deficient diet and exposed to nitrate or nitrite. In previous studies (Hong & Chow, 1988; Hong & Chow, 1987; Chow *et al*., 1984), 40% of rats fed a diet deficient in Vitamin E and selenium died after exposure to 1000mg/kg sodium nitrite. No fatalities were recorded in the control group, who were exposed to the same amount of nitrite but had Vitamin E or selenium-enriched diets (Hong & Chow, 1988; Hong & Chow, 1987; Chow *et al*., 1984). Because administration of Vitamin E and/or selenium drastically reduced mortality, the authors proposed that nitrate and nitrite toxicity is related to the generation of reactive oxygen species (ROS) (Chow & Hong, 2002). Specifically, they state that both nitrate and nitrite can arise from and create the nitric oxide radical ($NO^{\cdot}$). This radical may react with superoxide to form peroxynitrite ($ONOO^{-}$) , which is a highly reactive product. Other ROS may also be formed from interactions with nitrate and nitrite. Vitamin E is thought to reduce toxicity by reducing the production and availability of superoxide and $NO^{\cdot}$; selenium may inactivate $ONOO^{-}$. The authors conclude that nitrate and nitrite toxicity is primarily due to the formation of reactive oxygen species, and that proper supplementation with anti-oxidants or ROS-inactivating substances may reduce this toxicity (Chow & Hong, 2002).

Support for a role of nitric oxide in human nitrate metabolism was provided via experiments conducted by McKnight *et al*. (1997). In this investigation, ten healthy

human volunteers were monitored for intragastric nitric oxide (NO) production after consuming either a 2 mmol potassium chloride (control) solution or a 2 mmol potassium nitrate (experimental) solution. These concentrations were chosen to approximate the adult daily intake of nitrate in the study area. Samples of blood, gastric headspace gas, gastric juice, and saliva were taken every 20 minutes for 2 hours after consumption of the control or experimental solution, and every 30 minutes thereafter for a total experimental time of 6 hours. A nasogastric tube was placed in each subject to permit collection of headspace gas and gastric juice. Each volunteer was tested with both the control and experimental solutions, with at least one week in between tests.

After both control and experimental results were analyzed, a highly significant ($p<0.0001$) increase in gastric NO concentration was observed 60 minutes after potassium nitrate ingestion. This increase was not observed in subjects who received the control solution. Nitric oxide concentrations remained elevated for the remainder of the 6 hour study compared to control values. Significant increases in gastric, plasma, and total salivary nitrate were also observed. These results support the hypothesis that orally ingested nitrate can result in NO production in human subjects. However, it is important to note that the authors believe that NO production is beneficial, not toxic. They hypothesize that gastric NO plays an important role in killing ingested pathogenic microbial organisms, and can help maintain an antimicrobial environment in the stomach (McKnight *et al*., 1997).

Iijima *et al*. (2002) also performed experiments in humans to determine if nitric oxide was generated after oral administration of nitrate. Using a custom-made sensor probe,

these researchers monitored stomach and esophageal concentrations of NO in fifteen healthy volunteers after the instillation of 2mmol of potassium nitrate. The concentration of nitrate was chosen because it was approximately equivalent to the nitrate in a portion of salad. The probe was placed into the stomach before the introduction of nitrate, then withdrawn 1cm every two minutes to take additional real-time readings of the NO concentration. Nitric oxide concentration increased significantly compared to baseline after the ingestion of nitrate, particularly at the gastroesophageal junction. This increase was not observed after instillation of a nitrate-free water bolus. The authors state that the nitric oxide concentrations are highest at the gastroesophageal junction because it is the point where the pH decreases to that found in the stomach; thus, it is the location where nitrite is reduced to nitric oxide. Because the reaction is so rapid, the concentration of nitric oxide is highly localized. Iijima *et al*. (2002) postulate that this phenomenon may explain the high incidence of mutagenesis, neoplasia, and epithelial damage at this location; the concentration of nitric oxide produced may be mutagenic. Furthermore, they state that ascorbic acid and other antioxidants aid in the reduction of nitrite to nitric oxide, and thus may actually contribute to the mutagenic effects of nitrate/nitrite. This finding is in direct contrast to Chow & Hong (2002), who found a protective effect of antioxidant supplementation on nitrate/nitrite toxicity. Based on this data, the researchers conclude that ingested nitrate is reduced to nitrite in the oral cavity and swallowed into the stomach (see section 2.8.1). This nitrite then reacts with gastric juice and ascorbic acid (or other antioxidants) to form potentially mutagenic concentrations of nitric oxide at the gastroesophageal junction (Iijima *et al*., 2002).

A similar, but slightly different mechanism of nitrate/nitrite toxicity has been postulated by Titov and Petrenko (2005). Through kinetic experiments studying nitrite-induced methemoglobin formation in rabbit hemolysate and whole erythrocytes, the effects of nitrate at the cellular and molecular level were examined. While these researchers agree that a reactive oxygen species (ROS) is generated from nitrate/nitrite and acts to oxidize hemoglobin into methemoglobin, they suggest that $NO_2^{\cdot}$ is the primary reactive species generated. This differs from the conclusions of other authors, who suggest that $ONOO^-$ (Chow & Hong, 2002) or NO (Iijima *et al*., 2002) is responsible for the observed toxicity. Titov and Petrenko (2005) also state that nitric oxide generation is a "side reaction" of nitrate/nitrite metabolism, and not a major product of the reaction. Other authors (McKnight *et al*., 1997; Iijima *et al*., 2002) disagree, stating that NO is a significant product of nitrate/nitrite metabolism. At present, it appears that more study is required before the mechanism of nitrate/nitrite toxicity can be fully elucidated.

Despite decades of study, the pharmacokinetics and molecular mechanisms of nitrate toxicity remain contentious issues. Multiple studies have been performed in both animals and humans, yet each research group appears to interpret the results in a slightly different manner. Although many groups agree that reactive oxygen species play a role in nitrate toxicity, the identity of the specific molecule remains the subject of debate. Additional research is required to identify the compound or compounds responsible, and to clarify what role, if any, they play in human disease.

2.9 Introduction to proteins examined in this thesis

2.9.1 Proliferating Cell Nuclear Antigen (PCNA)

Proliferating cell nuclear antigen (PCNA) is a 36-kDa protein highly conserved in eukaryotes (Bruck & O'Donnell, 2001; Bravo & Macdonald-Bravo, 1987). A trimeric protein with two domains per subunit, PCNA forms a ring with six-fold symmetry (Paunesku *et al*., 2001). Indeed, PCNA is considered a member of the ring-type polymerase sliding clamp family, which is vital in the replication of DNA (Bruck & O'Donnell, 2001). During replication, PCNA forms a circular structure around DNA to anchor DNA polymerase (Bruck & O'Donnell, 2001). This permits constant attachment of DNA polymerase to the replicating strand, and allows rapid, continuous DNA replication. The circular conformation of PCNA is required to prevent buildup of torsional stress arising from the rotation of DNA polymerase during the replication process (Majka & Burgers, 2004). Replication factor C, an ATP-dependent clamp loader complex, is required to permit the insertion of the DNA into the ring structure of PCNA (Majka & Burgers, 2004; Bruck & O'Donnell, 2001). Logically, PCNA expression increases in S-phase, during DNA replication (Bravo & Macdonald-Bravo, 1987). Levels are highest in replicating cells and lowest in quiescent populations (Bruck & O'Donnell, 2001).

Proliferating cell nuclear antigen has many functions vital to cell maintenance and proliferation. The activity of PCNA is required for the simultaneous synthesis of both leading and lagging strands in DNA (Tsurimoto, 1998). The presence of PCNA also allows the replication of long DNA strands; without it, DNA polymerase would dissociate after replicating only a few bases (Tsurimoto, 1998). A vital role is played by

PCNA in DNA repair processes, specifically nucleotide excision repair, base excision repair, mismatch repair, and double-strand break repair (Maga & Hübscher, 2003). It may also assist in the maintenance of proper genomic methylation, chromatin cohesion, and chromosome structure. Due to interactions with proteins that control cell cycle regulation, PCNA is required for progression through the cell cycle (Paunesku *et al*., 2001; Tsurimoto, 1998). If PCNA levels are low, proliferation will not occur and the cell will enter the apoptotic pathway (Paunesku *et al*., 2001).

Levels of PCNA may have implications for human health. Overexpression of PCNA is the most common change found in tumour cells compared to normal control cells, especially in tumour cells with increased resistance to radiation. Therefore, PCNA expression is used to monitor the cell cycle time in tumour cells (Paunesku *et al*., 2001). Clinically, PCNA is often used to predict tumour growth and patient prognosis, but measures of proliferation alone may not provide an accurate measurement of cancer severity (Quiñones-Hinojosa *et al*., 2005). The long half-life of PCNA (20 hours) and variations in immunostaining intensity can make accurate measurements of cell proliferation difficult. Also, potential cross-reactivity may exist between PCNA antibodies and components of tumorigenic cells; some tumours score high on the PCNA immunolabelling index, but not for other indices of cell proliferation. This suggests that the PCNA antibody may cross-react with other cell components, which makes accurate measurement of proliferation very difficult (Quiñones-Hinojosa *et al*., 2005). Tachibana *et al*. (2005) suggest that PCNA may not be a reliable biomarker of proliferation since it is also involved in cell repair processes.

The expression of PCNA will be observed in this thesis for several reasons. First, monitoring levels of PCNA will indicate the effect that nitrate treatment has on the proliferation of HepG2 and HEK293 cells at the level of the protein. Because PCNA is known to play a role in DNA repair, changes in PCNA expression may indicate if nitrate exposure is causing DNA damage in these cell lines. Likewise, because low PCNA levels may lead to apoptotic cell death, monitoring PCNA may indicate the apoptotic status of exposed cells. Although PCNA levels may not be a completely reliable clinical tumour biomarker, large changes in PCNA *in vitro* may indicate a potential for carcinogenesis/tumorigenesis. Many epidemiological studies have examined the relationship between nitrate exposure and cancer risk; these experiments will attempt to characterise the relationship at the level of the protein. The expression of PCNA will also be examined to determine if previously documented increases in mRNA levels after nitrate exposure (Bharadwaj *et al.*, 2005) are also seen at the level of translation.

2.9.2 Heat Shock Protein 70 (Hsp70)

Heat shock protein 70 (Hsp70) is actually a family of highly-conserved, abundant proteins found in the cytosol and organelles of virtually all cells. These proteins consist of two subunits which total 70 kilodaltons (kDa): a 45kDa N-terminal ATPase domain, and a 25kDa C-terminal substrate-binding domain (Mayer & Bukau, 2005). The Hsp70 family includes inducible Hsp70, which will be examined in this thesis. Heat shock proteins are expressed in response to cellular stress, and perform many important intracellular functions. These include the folding of newly-synthesized proteins, the refolding of misfolded or aggregated proteins, the transport of proteins across membranes, and the control of regulatory protein activity. The Hsp70 proteins may also

help prepare damaged proteins for degradation. The “chaperone”, or protein-binding, activity of Hsp70 is ATP-dependent, and works together with co-chaperones (i.e. J-domain proteins, Bag proteins) and other co-operating chaperone systems (Mayer & Bukau, 2005).

Heat shock protein 70 has been shown to play an important role in human health. It has the ability to prevent both caspase-dependent and caspase-independent apoptosis, conferring a pro-survival effect (Parcellier *et al*., 2003). Unfortunately, Hsp70 is often overexpressed in cancer cells and malignant tumours, greatly increasing resistance to treatment. High Hsp70 expression may indicate a poor prognosis in several cancers (Jäättelä, 1999). However, this overexpression may itself be a useful therapeutic target; it may be possible to target cells overexpressing Hsp70 on the cell surface (Gehrmann *et al*., 2005). Conversely, administration of Hsp70 might also confer a protective effect in degenerative diseases through its pro-survival effect. Hsp70 therapy has been proposed to treat neurodegenerative diseases such as Alzheimers, Parkinsons, Huntingtons, and prion diseases (Sõti *et al*., 2005; Mayer & Bakau, 2005).

Several studies have examined the expression of Hsp70 in HepG2 cells. Salminen *et al*. (1996) exposed HepG2 cells to sub-lethal concentrations of hepatotoxic substances to determine if increases in Hsp70 protein or mRNA occurred. After exposure to bromobenzene, cadmium, cyclophosphamide, and diethylnitrosamine, Hsp70 protein and mRNA levels increased. However, no increase in Hsp70 was seen after exposure to carbon tetrachloride or cocaine. The authors hypothesize that Hsp70 induction is caused by the formation of protein and reactive metabolite adducts, and the lack of response

observed may be due to the inability of HepG2 cells to metabolically activate those substances. Alternatively, the authors propose that a different mechanism of injury, which does not involve Hsp70, may also be occurring (Salminen *et al*., 1996). HepG2 cells experienced a small increase in levels of Hsp70 protein when exposed to sublethal concentrations of nickel and cadmium in an experiment by Delmas *et al*. (1996). Elevated levels of Hsp70 protein were also found in HepG2 cells subjected to a one hour incubation at 41.8°C (Schueller *et al*., 2001). However, untreated HepG2 cells were also shown to express inducible Hsp70 protein, albeit at a lower concentration. Although Hsp70 has been associated with apoptosis in previous experiments, the expression of Hsp70 in the experiment by Shueller *et al*. (2001) was associated with the populations of both apoptotic and necrotic HepG2 cells. These experiments show that HepG2 cells are capable of experiencing a Hsp70-mediated stress response, but that not all hepatotoxic substances can produce this effect.

Inducible Hsp70 will be examined in this thesis as an indicator of the "classical" cellular stress response. By monitoring levels of Hsp70 protein in human cell lines after exposure to nitrate, it may be possible to determine the intracellular mechanisms involved in nitrate toxicity.

2.9.3 Heat Shock Cognate Protein 70 (Hsc70)

Heat shock cognate protein 70 (Hsc70) and Hsp70 (see 2.9.3) have many similarities. Like Hsp70, Hsc70 is a member of the Hsp70 family of proteins. Both isoforms migrate from the cytosol to the nucleus during heat shock in order to complex with vulnerable proteins (Gething & Sambrook, 1992), and both bind proteins in the same way

(Angelidis *et al*., 1999). Overexpression of Hsc70 and Hsp70 is seen in many tumours and tumour cell lines (Hfaiedh *et al*., 2005), and both proteins are considered to be future therapeutic targets (Brodsky & Chiosis, 2006). Both proteins also assist in the degradation of damaged or improperly folded intracellular proteins (Goldfarb *et al*., 2006). Because of their many similarities, Hsc70 and Hsp70 are considered to be "homologous but not identical" by Goldfarb *et al*. (2006).

However, there are several important differences which distinguish each isoform from the other. The Hsc70 protein is constitutively expressed in cells, while expression of Hsp70 is normally triggered by stress. In fact, Hsc70 is the only cytosolic Hsp70 family member which is ubiquitously and strongly expressed in all cells (Rohde *et al*., 2005). The Hsc70 protein has some distinct functions which Hsp70 does not share, such as catalyzing the ATP-dependent uncoating of clathrin-coated pits (Goldfarb *et al*., 2006). Experimental evidence suggests that the two proteins behave differently *in vitro*; Hsc70 appears to aggregate differently than Hsp70 when exposed to high temperatures in extracts of transfected monkey kidney CV1 cells (Angelidis *et al*., 1999). In HepG2 cells, the expression of Hsc70 did not increase when proteasomes were deactivated; however, the expression of Hsp70 increased three-fold (Liao *et al*., 2006). In experiments performed in yeast and plant models, mammalian Hsc70 was able to sustain growth alone, but mammalian or native Hsp70 alone was not. Subsequent molecular studies found significant differences in the ATPase and C-terminal domains of the two isoforms (Tutar *et al*., 2006). These results suggest that, despite their many similarities, Hsc70 and Hsp70 have distinct and important intracellular roles.

The expression of Hsc70 will be examined in this thesis in order to determine the effect of nitrate exposure on constitutively expressed stress proteins in exposed cells. Monitoring Hsc70 expression will also allow for a more complete examination of Hsp70 expression, as the relative changes may be compared. Because Hsc70 also moderates a distinct set of cellular processes, insight may be gained into how nitrate affects these processes.

2.9.4 Vascular Endothelial Growth Factor (VEGF)

Like Hsp70, vascular endothelial growth factor (VEGF) is actually a family of closely-related proteins with similar functions. The VEGF proteins have a homology domain of eight characteristically spaced cysteine residues and bind to VEGF-specific tyrosine kinase receptors (Roy *et al*., 2006). In order to become active, 21kDa VEGF monomer proteins must form a 42kDa homodimer (Voelkel *et al*., 2006). The VEGF proteins play a role in beneficial physiological processes such as embryonic development, wound healing, and compensatory angiogenesis in cardiovascular disease. However, VEGF is also implicated in the development of endometriosis, retinopathy, rheumatoid arthritis (Roy *et al*., 2006), and several lung function diseases (Voelkel *et al*., 2006). In this thesis, VEGF-A, the best-known member of the VEGF family, will be examined. For the sake of clarity, VEGF-A will simply be referred to as VEGF.

The best-known activity of the VEGF protein is its pro-angiogenic function. This has obvious ramifications for human health, particularly cancer development. As tumours grow, the tissue becomes hypoxic. Hypoxia is a trigger for VEGF production, which triggers angiogenesis and encourages tumour growth (Ribatti, 2004). Vascular

endothelial growth factor is overexpressed in many *in vivo* tumours and *in vitro* tumour cell lines; several studies have shown that cancer patients possess higher VEGF serum levels than normal subjects (Ribatti, 2004). Increased VEGF expression has been correlated with severity and treatment resistivity in hepatocellular carcinoma (Tanaka & Arii, 2006; Pang & Poon, 2006). Upregulation of VEGF is also a common clinical finding in renal cancer (Nathan *et al*., 2006).

However, VEGF may also aid in the progression and development of cancer by other means. By increasing vessel permeability, neovascularization, and lymphangiogenesis, VEGF may also encourage tumour growth and metastasis (Roy *et al*., 2006). An autocrine feedback loop involving VEGF may be responsible for the growth of breast cancer. Mercurio *et al*. (2005) hypothesize that tumour cells possess VEGF receptors, and that VEGF produced by surrounding tumour cells binds to and activates these receptors. This triggers a pro-survival, anti-apoptotic signalling cascade that confers increased resistance and survival to affected cells. In order to combat the harmful effects of VEGF in cancer, a VEGF monoclonal antibody (bevacizumab, Avastin™) has been developed and approved to treat several types of cancer (Roy *et al*., 2006). However, more research is required to fully elucidate and prevent the role of VEGF in cancer development.

The expression of VEGF will be examined in this thesis because of its importance to cancer progression and development. The relationship between nitrate and cancer is unresolved at the epidemiological level; these experiments will provide some insight at the molecular level in human cell lines. Also, VEGF appears to activate nitric oxide

(NO) synthase in endothelial cells, which produces NO (Voelkel *et al*., 2006). Breakdown products of NO include nitrate and nitrite, which may add to the total body burden. By examining the effect of nitrate on VEGF expression, it may be possible to determine if nitrate affects cancer progression through a pro-angiogenesis pathway. Also, in previous studies, VEGF mRNA expression was shown to decrease in HepG2 cells exposed to nitrate (Bharadwaj *et al.*, 2005). This study will examine the expression of VEGF protein to determine if this downregulation extends to the level of translation.

2.10 Relevance to current knowledge: cytotoxicity and protein expression

The majority of research to date on nitrate toxicity has been conducted on experimental animals, with results extrapolated to predict human toxicity. Many of these studies have been conducted *in vivo*, with focus on organ or system toxicity. However, very few studies have examined the effect of nitrate exposure at the cellular level, particularly *in vitro* cultured human cell lines.

The first paper to describe the effects of nitrate toxicity on human cell lines, using measures of both cytotoxicity and changes in gene expression, was a study by Bharadwaj *et al*. (2005). In the cytotoxicity portion of the study, HepG2 cells were exposed to potassium nitrate for 24 hours at concentrations between 3 mg/L and 1000 mg/L (29.7 µM to 9.9 mM). These concentrations were chosen because they reflect environmentally relevant concentrations of nitrate (Bharadwaj *et al.*, 2005). Subsequent to treatment, the exposed cultures were analyzed for cell viability (Neutral Red assay) or proliferation (5-bromo-2'-deoxyuridine (BrdU) assay). Significant reductions in proliferation compared to control levels were observed in all nitrate treatment groups.

No significant decrease in cell viability was observed, suggesting that cell proliferation was a more sensitive marker of nitrate toxicity at these concentrations. These results also indicate that these environmentally relevant concentrations of nitrate do not exert lethal effects on HepG2 cells under these experimental conditions.

In the gene expression portion of the study by Bharadwaj *et al*. (2005), HepG2 cells were exposed to potassium nitrate at concentrations of 3 mg/L, 45 mg/L, 150 mg/L, or 500 mg/L for 24 hours. Total RNA was extracted, and the technique of reverse transcription was used to form target cDNA. Control and experimental cDNA samples were labelled with different fluorescent markers and hybridized to a custom-made human cDNA microarray chip. During analysis of the chip with a scanning laser fluorescence confocal microscope, an image of the fluorescence from each marker was created. These images were then merged and analyzed to determine the relative intensity of each marker at each location on the chip. Based on the relative intensity of each fluorescent marker, conclusions were made about the degree of expression of each gene on the chip. After eliminating all genes that did not experience at least a two-fold change in expression, it was concluded that 58 genes experienced a two-fold change in expression after nitrate exposure, and 80 genes experienced a three-fold change. The proportion of up- and down-regulated genes was approximately equal. A wide variety of genes were affected by nitrate exposure, including those involved in cell cycle control, DNA repair, immune response, and stress response (Bharadwaj *et al.*, 2005). This indicated that the cellular response to nitrate exposure was complex, involving genes responsible for important cellular functions.

A later study, performed by Jondeau *et al*. (2006), provided additional data by examining the sublethal cytotoxicity of sodium nitrate on HepG2 cells, at concentrations of 14.6-257 mM. The cytotoxicities of ten other contaminants (acrylamide, atrazine, benzo[a]pyrene, 17-α-ethinyloestradiol, 4-octylphenol, hydrogen peroxide, sodium bromate, sodium chlorate, bisphenol-A, and 2,4 diaminotoluene) were also determined. HepG2 cells were exposed to $NaNO_3$ for 20 hours before analysis. Three different cytotoxicity assays were utilized, and the relative sensitivity of each was determined. Each assay examined a different indicator of cytotoxicity: rate of RNA synthesis, ATP abundance, or metabolic activity. Toxicity was defined as an IC_{50} value, which represented the concentration of the test compound which "inhibit[ed] response assay by 50% compared to the solvent control" (Jondeau *et al.*, 2006). Of the 11 contaminants tested, sodium nitrate was shown to be the least toxic in both the RNA and metabolic assays, and slightly more toxic than only one compound (sodium chlorate) in the ATP assay. All assays produced a strong sigmoidal concentration-response curve when assay response was plotted as a function of logarithmic nitrate concentration. The IC_{50} values for sodium nitrate toxicity in HepG2 cells in these experiments were 40.6 mM (~3451 mg/L) in the RNA assay, 160 mM in the metabolic assay (~13598 mg/L), and 72.8 mM (~6187 mg/L) in the ATP assay. This indicates both a low acute toxicity of sodium nitrate, as well as the potential for considerable variation between different types of cytoxicity assays. The authors state that the relatively low cytotoxicity of sodium nitrate is consistent with the results from the study by Bharadwaj *et al*. (2005).

The research described in this thesis will expand upon the early studies by Bharadwaj *et al*. (2005) and Jondeau *et al*. (2006) by further characterizing the cytotoxic effects of

nitrates on human cell lines. Additional information about the contribution of different nitrate salts to cytotoxicity will be obtained through the use of potassium nitrate, ammonium nitrate, and sodium nitrate in these experiments. The effect of exposure time on the severity of cytotoxicity will also be examined through the use of several timepoints for each assay. To better elucidate possible tissue specificity effects of nitrate, cytotoxicity and protein expression assays will be conducted with both the HepG2 cell line and a human embryonic kidney cell line (HEK293). These experiments appear to be the first to use HEK293 cells to determine the toxicity of nitrate. This research is also unique because it appears to be the first investigation of the effect of nitrate exposure on protein expression in human cell lines. This will expand upon the work of Bharadwaj *et al*. (2005) by examining if the nitrate-induced differences in transcription (RNA) are also seen at the level of translation (protein). By expanding upon current research, this thesis will fill important knowledge gaps and provide greater understanding of the effects of nitrate exposure at the cellular and molecular level.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study design

The purpose of this study was to determine the effects of environmentally relevant nitrate exposure on human cells in an *in vitro* system. This was achieved through the use of two cell lines: HepG2, a human hepatocarcinoma line; and HEK293, a human embryonic kidney line. These cell types were chosen to represent the liver and kidney, two possible target organs of nitrate toxicity. Each experiment contained a control group, which was not exposed to nitrate, and experimental groups which were exposed to different concentrations of nitrate. Generally, each experiment was performed with each nitrate salt, more than one exposure time (24, 48, and/or 72 hours), and each cell type. Each combination of nitrate salt, exposure time, and cell type was repeated three times, although exceptions occurred and will be discussed within individual assay descriptions.

The nitrate concentrations used in these assays may be found in Table 3.1; they were chosen to represent concentrations below the current regulatory limit (45 mg/L NO_3^-), as well as those which were likely to induce cytotoxicity. By using these concentrations, the amount of nitrate exposure required to cause overt cytotoxicity as well as more subtle changes in cell function could be determined. These concentrations are expressed in molar units in Table 3.2. In order to determine if different salts of nitrate affected toxicity, experiments were conducted with potassium and ammonium nitrate. Additional

experiments were conducted with alternate salts of potassium, ammonium, and nitrate to determine the relative contribution of each ion to overall toxicity.

To determine the effects of nitrate exposure on human cell lines, both cytotoxicity and protein expression assays were used. Cytotoxicity assays included the Neutral Red assay for cell viability, and the 5-bromo-2'-deoxyuridine enzyme-linked immunosorbent assay (BrdU ELISA) for proliferation. Protein expression was determined through Western blotting. In the Neutral Red assays, each nitrate treatment was represented by one culture flask. The control group was also represented by one flask. This experimental unit (the set of experimental flasks plus the control flask) was then replicated a total of three times in order to give three measurements of cell viability for each concentration. These were pooled and used for analysis. For the BrdU ELISA assay, each treatment was administered to 6 wells of a 96-well plate. Three replicate 96-well plates were analyzed for each combination of cell type, nitrate salt, and exposure period. However, due to difficulties in pooling data from the three plates, a representative dataset was chosen for analysis and graphing. The Neutral Red and BrdU ELISA assays were performed on cells treated with KNO_3 and NH_4NO_3 as well as the alternate salts CH_3COOK, CH_3COONH_4, and $NaNO_3$ (CH_3COOK assays were only performed in HepG2 cells). An additional viability assay, the resazurin (alamar blue) assay, was performed on HepG2 and HEK293 cells exposed to NH_4NO_3 for 24 hours. This assay was performed in a single 96-well plate with each treatment replicated in three plate wells.

The exposure periods used varied between assays. The Neutral Red assays performed with KNO_3 or NH_4NO_3 used exposure periods of 24, 48 (HepG2 cells only), and 72

hours. However, the alternate salt Neutral Red assays used only a 48 hour exposure period. The BrdU ELISA assays performed with KNO_3 or NH_4NO_3 used exposure periods of 24 or 72 hours, but the alternate salt BrdU ELISA assays used only 48 hour exposure periods. The one resazurin assay performed used an exposure time of 24 hours.

For protein expression assays, two successful Western blots were obtained for each protein of interest. However, the VEGF protein visualized poorly so no data were available for HepG2 expression and only one blot was performed for the HEK293 exposures. Both HepG2 and HEK293 cell lines were exposed to potassium or ammonium nitrate for 24 hours. Using mouse monoclonal antibodies, each combination of cell type and nitrate salt exposure was examined for expression of PCNA (#sc56, Santa Cruz Biotechnology, Santa Cruz, CA), Hsp70 (#SPA-810, Stressgen Bioreagents, Victoria, BC) and HSC70 (#sc7298, Santa Cruz Biotechnology, Santa Cruz, CA). VEGF (#sc7269, Santa Cruz Biotechnology, Santa Cruz, CA) expression was examined only in HEK293 cells, and only one blot of potassium and ammonium exposure could be obtained due to extremely low VEGF expression. All antibodies utilized were IgG_{2a} mouse monoclonal antibodies, with the exception of the Hsp70 antibody (IgG_1). A secondary goat anti-mouse antibody, conjugated with horseradish peroxidase (#170-6516, Bio-Rad Laboratories, Mississauga, ON), was used for visualization of the protein bands.

Table 3.1: Concentrations of experimental and alternate salt treatment solutions used in cytotoxicity and protein expression studies

Neutral Red Assay	BrdU Assay	Resazurin Asssay	Protein Expression
mg/L	mg/L	mg/L	mg/L
0	0	0	0
500	0.001	3	3
1000	0.05	45	45
1500	3	150	150
2000	10	500	500
2500	45	1000	
3000	150	1500	
3500	250	2000	
4000	500	2500	
4500	1000	3000	
5000	1500	3500	
	2000	4000	
	2500	4500	
	5000	5000	

Note: the HepG2 alternate salt Neutral Red assays (48 hour CH_3COOK, CH_3COONH_4, and $NaNO_3$) included nitrate concentrations of 3, 45, and 150 mg/L as well as the other nitrate concentrations listed above.

Table 3.2: Concentrations of experimental and alternate salt treatment solutions expressed in molar units

Substance	0.001 mg/L	0.05 mg/L	3 mg/L	10 mg/L	45 mg/L	150 mg/L	250 mg/L	500 mg/L	1000 mg/L	1500 mg/L	2000 mg/L	2500 mg/L	3000 mg/L	3500 mg/L	4000 mg/L	4500 mg/L	5000 mg/L
KNO_3 101.1 g/mol	9.89 nM	0.49 µM	29.7 µM	98.9 µM	0.45 mM	1.5 mM	2.5 mM	4.9 mM	9.9 mM	14.8 mM	19.8 mM	24.7 mM	29.7 mM	34.6 mM	39.6 mM	44.5 mM	49.5 mM
NH_4NO_3 80.04 g/mol	12.5 nM	0.62 µM	37.5 µM	125 µM	0.56 mM	1.9 mM	3.1 mM	6.2 mM	12.5 mM	18.7 mM	25.0 mM	31.2 mM	37.5 mM	43.7 mM	50.0 mM	56.2 mM	62.5 mM
$NaNO_3$ 84.99 g/mol	11.8 nM	0.59 µM	35.3 µM	118 µM	0.53 mM	1.8 mM	2.9 mM	5.9 mM	11.8 mM	17.6 mM	23.5 mM	29.4 mM	35.3 mM	41.2 mM	47.1 mM	53.0 mM	58.8 mM
CH_3COOK 98.15 g/mol	10.2 nM	0.51 µM	30.6 µM	101.9 µM	0.46 mM	1.5 mM	2.5 mM	5.1 mM	10.2 mM	15.3 mM	20.4 mM	25.5 mM	30.6 mM	35.7 mM	40.8 mM	45.9 mM	50.9 mM
CH_3COONH_4 77.08g/mol	13.0 nM	0.65 µM	38.9 µM	130 µM	0.58 mM	1.9 mM	3.2 mM	6.5 mM	13.0 mM	19.5 mM	25.9 mM	32.4 mM	38.9 mM	45.4 mM	51.9 mM	58.4 mM	64.9 mM

3.2 Cell lines and culture conditions

Human hepatocarcinoma (HepG2) and human embryonic kidney (HEK293) cell lines were used in these experiments. Both cell lines were obtained from the American Type Culture Collection (HepG2: HB-8065; HEK293: CRL-1573). These cell lines were chosen because they represent two potential target organs for nitrate toxicity. As previously mentioned, the rapid absorption of nitrate from the intestine and its systemic distribution result in extensive hepatic exposure, and the excretion of nitrate and related products in the urine (Bartholomew & Hill, 1984) suggest that renal cells may also be susceptible to toxic insult. The addition of HEK293 cells to the experimental protocol will allow comparison of the results obtained from Dr. Bharadwaj's previous work with HepG2 cells (Bharadwaj *et al.*, 2005), and provide information on potential tissue specificity of the resulting toxicity. The HepG2 cell line is considered an appropriate model for human toxicity studies (Jiménez *et al*., 2002; Wilkening *et al.*, 2003; Seth *et al*., 2004), particularly genotoxicity studies (Knasmüller *et al*., 2004). The HEK293 cell line has also been used as a model for human toxicity (Zheng *et al*., 2003; Kirkpatrick *et al*., 2003), and have been used in transcriptomic/proteomic studies (Prat *et al*., 2005). Given their widespread use in toxicity testing, particularly transcriptomic/toxicogenomic studies, HepG2 and HEK293 are suitable cells for these experiments.

Cells were cultured at 37°C and 5% CO_2 in a humidified incubator in 100x20mm plates (BD Falcon™, #353003, Franklin Lakes, NJ). Each cell type was grown in monolayer cultures, in a solution of Minimum Essential Medium (Gibco, #41500-034), supplemented with 1M sodium bicarbonate, 1% sodium pyruvate, 10% fetal bovine serum, and 1% penicillin/streptomycin (see section 3.3 for more detail). Culture medium

was renewed approximately every 48 hours. Cell cultures were passaged (subcultured) once confluence reached 80-85%, usually in approximately one week for HepG2 cells and four days for HEK293 cells. Experiments utilized HepG2 cells from passages 10-20; attempts were made to perform similar experiments with similar passage numbers and to keep passage numbers as low as possible. Some evidence has suggested that HepG2 cells experience changes in gene expression when repeatedly subcultured (Wilkening & Bader, 2003). HEK293 cells were used from passages 2 to 12, again utilizing similar passage numbers for each experiment where possible and keeping the number of passages to a minimum.

All cell cultures and associated materials were assumed to have a rating of Biosafety Level 2, and were handled and disposed of in accordance with University of Saskatchewan Department of Health, Safety, and Environment regulations.

3.3 Media preparation

Pre-packaged Minimum Essential Medium (MEM) powder (Gibco, #41500-034) was transferred to a 2 L volumetric flask with the aid of a funnel. Double-distilled water was added to reach a final volume of 2 L, and the solution was mixed for at least two hours on a magnetic stirrer. The solution was then vacuum filtered into sterile bottles. A 1 M sodium bicarbonate and phenol red solution was added to act as a buffer and pH indicator. A 1% 100 mM sodium pyruvate solution was also added to each bottle of media. Immediately before use in cell culture, the media was supplemented with 10% fetal bovine serum (Sigma, #F-1051, Oakville, ON) and 1% penicillin (10,000 units/mL)/streptomycin (10,000µg/L) (Gibco, #15140-122). This step was performed

immediately before use to prevent protein degradation. Bottles of media were stored at 4°C to prevent bacterial growth.

The experimental solutions were formulated by dissolving each powdered or crystalline salt into culture medium to create a stock solution of 50000 mg/L. The origin of each salt was as follows: KNO_3 (#ACS687), NH_4NO_3 (#ACS078), $NaNO_3$ (#ACS825), and CH_3COONH_4 (#ACS036) were all obtained from BDH Inc. (Toronto, ON), while CH_3COOK (#P1190) was obtained from Sigma (Oakville, ON). In order to prevent bacterial contamination, the well-mixed stock solution was passed through a 0.2 µm syringe filter (Acrodisc® 25mm filter, #4612, Pall Corporation, Mississauga, ON) into a sterile centrifuge tube. Appropriate dilutions were obtained by mixing the stock solution with required volumes of cell culture medium in a new sterile centrifuge tube. Treatment solutions were stored at 4°C until required, and were allowed to reach room temperature before addition to cell culture flasks. Before use, treatment solutions were examined for indications of bacterial contamination, and were discarded if the solution was murky or discoloured. Solutions were not used if more than two months had elapsed since formulation; most solutions were replaced on a more frequent basis due to usage.

3.4 Cell harvesting and counting

The desired plate was removed from a 37°C + 5% CO_2 incubator and placed in a Level 2 Biosafety Cabinet. After aspirating and discarding the media, the cells were washed with a 5 mL aliquot of phosphate-buffered saline (PBS). The PBS wash was then aspirated and discarded. Approximately 2.5 mL of trypsin + EDTA (#25200-72, Gibco, Burlington, ON) was added, and the plate was then returned to the incubator for 5-10

minutes, with monitoring to determine the degree of cell detachment. When sufficient cell detachment had been achieved, the plate was returned to the Level 2 Biosafety Cabinet. Approximately 5 mL of sterile media was added to discontinue trypsin activity. The cells were then resuspended by repeated pipetting and expulsion onto the bottom wall of the plate.

To count the cells, 10 µL aliquots of well-mixed cell suspensions were placed in a microcentrifuge tube (AdvanTech® #AD151-N, DiaMed Lab Supplies Inc., Mississauga, ON) An equal volume of trypan blue solution (0.4%, #15250-061, Gibco, Burlington, ON) was added, and the solutions were mixed by repeated pipetting. An incubation time of approximately 1 minute was permitted to allow staining of nonviable cells. The solutions were then dispensed onto a haemocytometer (#3336-A, Arthur H. Thomas Company, Philadelphia, PA) and the cells were counted. Non-viable cells were not included in the field counts. The average of five haemocytometer fields was calculated, and the number of cells per millilitre was determined using the equation:

$$cells/mL = field\,average \times 2 \times 10^4 \qquad (3.1)$$

The counted cells were then discarded.

3.5 Neutral Red cell viability assay

This assay was selected to compare the viability of cell cultures in the treatment groups to those in the control groups. The Neutral Red assay measures dye retention in viable cells; uncharged dye molecules move into cells via passive diffusion and become

trapped in the low-pH environment of the lysosome. After washing to remove excess dye, lysis buffer is applied and the absorbance of the lysate is measured at 450nm in a spectrophotometer (Rashid *et al*., 1991).

The HepG2 or HEK293 cells were harvested and counted as described in the cell harvesting protocol (see section 3.4). The cells were plated in Nunclon $25cm^2$ tissue culture flasks (Easy Flask, #156340, Nalge Nunc International, Rochester, NY) at a density of $1x10^6$ cells per 24 hour assay, $7.5x10^5$ cells per 48 hour assay, or $5x10^5$ cells per 72 hour assay. Culture medium was added to bring the flasks to a final volume of 4.5 mL. The flasks were incubated under 37°C and 5% CO_2 conditions overnight, then exposed the next day. A 0.5 mL aliquot of media-based treatment solution was added to the experimental groups, taking care not to dislodge cells from the flask. For control flasks, a 0.5 mL aliquot of media was added following the same procedure as the treatment group. The cells were then incubated for the required time period (24, 48, or 72 hours).

The day prior to the Neutral Red analysis, the Neutral Red assay solution (Sigma, #N2889, Oakville, ON) was removed from refrigeration at 4°C. An aliquot corresponding to the volume required for the next day's analysis was removed and passed through a 0.2 µm syringe filter (Acrodisc® 25mm filter, #4612, Pall Corporation, Mississauga, ON) in order to remove crystallogenic particles. The filtrate was then collected in a centrifuge tube (#21008-103, VWR, Edmonton, AB), and incubated under the same conditions as the experimental flasks. The cap of the centrifuge tube was not

fully tightened in order to allow the Neutral Red solution to equilibrate with the incubator conditions.

Two hours prior to the expiration of the exposure period, the flasks were removed from the incubator. An aliquot of the previously filtered Neutral Red dye, corresponding to 1/10 of the culture volume (i.e. 0.5 mL) was added in HepG2 cultures, taking care not to dislodge the cells from the flask. Because the HEK293 cultures experienced adverse effects from high concentrations of Neutral Red dye, a 1/100 (50 µL) aliquot of dye was used for these plates. The flasks were returned to the incubator for the remainder of the exposure period.

After the exposure period had expired, the flasks were removed from the incubator to the Level 2 Biosafety Cabinet. The supernatant was aspirated and discarded. Each HepG2 flask was washed three times with a 5 mL aliquot of phosphate-buffered saline (PBS); the supernatant was discarded after each wash. Care was taken to avoid application of the PBS directly onto the cells, in order to prevent detachment from the flask. However, due to the greater tendency of HEK293 cells to detach from the culture flasks, only two PBS washes of 2 mL each were used for the HEK293 plates. This allowed for adequate removal of excess dye but avoided the catastrophic cell loss which occurred if the HepG2 washing protocol was used. After the third wash supernatant had been removed, an aliquot of lysis buffer (50% ethanol + 1% acetic acid) equal to the original culture volume (i.e. 5 mL) was added. The flasks were then placed on a gyrorotary shaker and subjected to gentle agitation for 10 minutes.

After agitation, the lysate in each flask was added to a spectrophotometric cuvette (#37001-732, VWR, Edmonton, AB) and diluted 1:3 with additional lysis buffer (i.e. 0.5 mL cell lysate + 1 mL lysis buffer). The cuvettes were then read in a spectrophotometer (SmartSpec^{TM} Plus, #170-2525, Bio-Rad Laboratories, Mississauga, ON) at a wavelength of 540nm, with background subtraction of 690nm. These results were recorded, and the cell lysate was discarded.

3.6 5-bromo-2'-deoxyuridine Enzyme-Linked Immunosorbent Assay (BrdU ELISA)

This assay was selected to compare the degree of cell proliferation in the cell cultures exposed to treatment solutions to those in the control groups. 5-bromo-2'-deoxyuridine (BrdU) acts as a thymidine analogue and is incorporated into the DNA of replicating cells. After lysis and denaturation of the cells, an anti-BrdU antibody, conjugated to a peroxidase enzyme, is applied and binds to the BrdU present in the cells. After application of a substrate solution, the enzyme catalyzes a colourimetric reaction and the plates are read at 450nm in a plate reader (Cell proliferation ELISA, BrdU (colorimetric) Instruction Manual, Roche Applied Science, 2004).

This assay was performed according to instructions provided in the BrdU assay kit (Cell proliferation ELISA, BrdU (colorimetric), #11 647 229 001, Roche, Laval, QC). Cells were harvested and counted as described in the cell counting protocol (see section 3.4). Cell suspensions were plated into clear 96-well tissue culture plates (Nunclon Delta surface 96-well plates, #167008, Nalge Nunc International, Rochester, NY), giving a final density of 1000 cells per well for a 24 hour exposure, 500 cells per well for a 48 hour exposure, and 50 cells per well for a 72 hour exposure. All wells were normalized

to a final volume of 90 µL through the addition of media. The plates were then incubated in 37°C + 5% CO_2 conditions overnight.

The following day, 10 µL aliquots of the appropriate media-based treatment were introduced into each well of the plate. Control wells received a 10 µL aliquot of media. Wells were treated in groups of six, in order to minimize the effects of biological variation from well to well. The plates were then returned to the incubator for the appropriate exposure time (24, 48, or 72 hours).

Immediately prior to the expiration of the exposure time, the BrdU Labelling Solution (Bottle 1) from the kit was removed from the -20°C freezer and allowed to thaw at room temperature. Once thawed, the labelling solution was diluted 1:100 with sterile media, with 1 mL of diluted label required for each plate analyzed. After the diluted labelling solution was created, the 96-well plates were removed from the incubator into the Level 2 Biosafety Cabinet. 10 µL of diluted labelling solution was added to each well, with the exception of the label control wells; these wells received 10 µL of media only. The plates were then returned to the incubator for two hours, to allow for incorporation of the label into the DNA of proliferating cells. After the labelling incubation period had expired, the plates were removed to the Level 2 Biosafety Cabinet. The labelling medium in each well was then removed by suction. The plates were then stored at 4°C for a maximum of one week before further analysis.

The procedure was resumed by treating all wells with 200 µL of FixDenat (Bottle 2) solution, as provided in the kit. This reagent served to fix the cells and denature the

DNA in preparation for analysis. The plates were then incubated at room temperature for 30 minutes. Immediately prior to the expiration of the FixDenat incubation time, the anti-BrdU peroxidase (POD) solution (Bottle 3) was diluted 1:100 with antibody dilution solution (Bottle 4). 10 mL of diluted anti-BrdU POD solution was required for each plate analyzed. After the FixDenat incubation period had expired, the FixDenat solution was removed by shaking the plates into a sink and blotting onto a paper towel. Each well was then treated with 100 µL of the diluted anti-BrdU POD solution, and the plates were incubated at room temperature for an additional two hours.

Prior to the expiration of the anti-BrdU POD exposure period, the washing buffer concentrate (Bottle 5) was diluted 1:10 with double distilled water to form the washing buffer. Approximately 90 mL of diluted washing buffer was required for each plate analyzed. After the expiration of the anti-BrdU POD incubation time, the anti-BrdU POD solution was removed by shaking the plates into a sink and blotting onto a paper towel. Each well was then washed three times with the diluted washing buffer, with removal of the washing buffer by the shaking and blotting procedure in between each wash. After the washing procedure was completed, 100 µL of substrate solution (Bottle 6) was added to each well, and the plates were incubated at room temperature until a blue colour was visible in the wells. Once the blue colour was sufficient for development, 25 µL of 1 M H_2SO_4 was added to each well to serve as a stop solution.

Immediately after the addition of the stop solution, the plates were spectrophotometrically analyzed in a microplate reader (EL_X808 Ultra Microplate Reader, Bio-Tek Instruments) at a wavelength of 450nm, after gentle shaking for one

minute. These results were recorded using KC4 Kineticalc for Windows software version 3.3 (Bio-Tek Instruments Inc., Winooski, VT), and the plates were then discarded.

3.7 Resazurin cell viability assay

This assay, like the Neutral Red assay (see section 3.5), was selected to compare the viability of cell cultures in the treatment groups to those in the control groups. However, the resazurin assay indicates viability as a function of metabolic activity, not dye retention. Briefly, living cells reduce resazurin to resorufin, a fluorescent product. Resorufin may be detected by using an excitation wavelength of 560nm and a detection wavelength of 590nm. The strength of the fluorescent signal is considered proportional to the viability of cells in the sample (TOX-8 Bulletin, Sigma, 2007).

Cells were harvested and counted as described in the cell harvest protocol. The cell suspensions were plated into a single black 96-well plate (Nunclon Delta surface 96-well plates, #137101, Nalge Nunc International, Rochester, NY) at a density of 30 000 cells per well for a 24 hour exposure. The volume of all wells was then normalized to 90 µL by addition of sterile culture medium. The plate was then incubated in 37°C + 5% CO_2 conditions overnight.

The following day, a 10 µL aliquot of the appropriate media-based treatment was introduced into each well of the plate. Control wells received a 10 µL aliquot of media. Wells were treated in groups of three, in order to minimize the effects of biological variation from well to well. The plate was then returned to the incubator for 24 hours.

Two hours prior to the expiration of the treatment exposure period, the plate was removed from the incubator and placed into the Level 2 Biosafety Cabinet. Resazurin dye solution (#TOX-8, Sigma, Oakville, ON) was added to each well in a volume equal to 1/10 the culture volume (i.e. 10 µL). Fluorescence control wells received 10 µL of media instead of the resazurin solution. Care was taken to reduce the exposure of the treated plate to excessive light exposure. The plate was then returned to the incubator for an additional two hours to allow the reduction of the resazurin dye by living cells to its fluorescent product.

After the completion of the dye incubation period, the plate was removed from the incubator and wrapped in aluminium foil to prevent contact with light. The plate was then analyzed in a fluorescent plate reader (Fluorolite 1000, Dynex Technologies, Chantilly, VA), with an emission wavelength of 590nm and an excitation wavelength of 560nm. The plate was then discarded.

3.8 Protein extraction

Cells were harvested and counted as described in the cell harvest protocol (see section 3.4). The cell suspensions were plated into 100x20mm tissue-culture grade plates (BD Falcon™, #353003, Franklin Lakes, NJ) at a density of $4x10^6$ cells for a 24 hour exposure. Culture medium was then added to reach final plate volumes of 9 mL. The plates were then incubated in 37°C + 5% CO_2 conditions overnight.

The following day, a 1 mL aliquot of the desired treatment was introduced into each plate. Control plates received a 1 mL aliquot of media. The plates were then returned to the incubator for 24 hours.

Upon expiration of the treatment time, the plates were removed from the incubator and placed into the Level 2 Biosafety Cabinet. The combined media and treatment liquids were removed by suction, and plates were washed twice with 5 mL aliquots of PBS. Care was taken to avoid pipetting PBS directly onto the cells. After aspiration of the last PBS wash, 1 mL of extraction buffer (see Appendix A for recipe) was applied to each plate. Careful rotation of plates was performed to ensure all cells were exposed to the extraction buffer. The plates were then allowed to incubate at room temperature for approximately one minute. A sterile rubber policeman or cell scraper was used to dislodge the cells from each plate. The cell suspension was then pipetted into a microcentrifuge tube (AdvanTech® #AD151-N, DiaMed Lab Supplies Inc., Mississauga, ON) and immersed in ice to prevent protein degradation. Samples were then sonicated three times for ten seconds each, using a sonicator (Sonifer® cell disruptor #W140, Heat Systems-Ultrasonics Inc., Plainview, NY). Sonication permitted greater lysis of the cells for more complete protein extraction. Samples were re-immersed in ice after sonication.

Sonicated samples were then spun down for 15 minutes at 15 000 rpm in a 4°C centrifuge (Eppendorf 5403, Eppendorf Canada Ltd., Mississauga, ON). Supernatants were collected and partitioned into five microcentrifuge tubes for freezing. Extracts to be stored were briefly flash-frozen in liquid nitrogen, then placed in a -80°C freezer until required.

3.9 Protein quantification

The Bradford assay was used to quantify proteins for Western blotting. Concentrated Bradford reagent (5x) was removed from 4°C storage and diluted with sterile triple-distilled water. 1.995 mL of diluted Bradford reagent was pipetted into a plastic spectrophotometric cuvette (#37001-732, VWR, Edmonton, AB). Protein extracts were retrieved from -80°C storage and placed on ice. A 5 µL aliquot of the desired extract was introduced into each cuvette, yielding a final cuvette volume of 2 mL. Cuvette contents were thoroughly mixed by repeated pipetting. Cuvettes were allowed to incubate at room temperature for five minutes to permit sufficient time for the reaction to occur. Cuvettes were read in a spectrophotometer (SmartSpec™ Plus, #170-2525, Bio-Rad Laboratories, Mississauga, ON) using the Bradford protein assay protocol. A calibration curve was used to quantify protein concentrations. The resulting concentrations were recorded for each sample, and volumes equivalent to 10 µg of protein were calculated. Protein samples were then returned to -80°C storage.

3.10 Western Blotting

The Western Blotting technique was used to separate, identify, and qualitatively assess protein concentrations from exposed and control cells. For gel casting and electrophoresis steps, the Mini-PROTEAN®-3 system (#165-3301 & 165-3302, Bio-Rad Laboratories, Mississauga, ON) was used.

Protein separations were performed on 0.75 cm thick 10% SDS-PAGE gels. Running gel solutions were mixed and then pipetted into 0.75 cm thick glass gel-casting plate assemblies. Immediately after the running gels were poured, small amounts of

isobutanol were pipetted onto the running gels in order to produce a smooth surface. Once the running gels had polymerized, the isobutanol layer was discarded. Stacking gel solutions were mixed and pipetted on top of the running gel layer. Immediately after pouring the stacking gels, 0.75 cm 10-well combs were pushed into the stacking gel liquid to produce wells. Combs were removed after the stacking gels had polymerized. The gels were removed from the casting apparatus and were placed into an electrophoresis apparatus.

Protein extracts were removed from -80°C storage and placed on ice. Using concentrations determined by the protein quantification procedure (see section 3.9), volumes equivalent to 10 µg of protein for each sample were placed in microcentrifuge tubes. Aliquots of SDS-PAGE loading dye were added to each tube to permit visualization of the protein solution. After boiling in a 98°C water bath for 10 minutes, samples were spun down in a centrifuge at maximum velocity for approximately 10 seconds to allow for settling of extraneous material. The samples were then pipetted into the appropriate well of the prepared SDS-PAGE gels. Unused wells were filled with 5 µL of 1x SDS-PAGE loading dye. A 5 µL aliquot of pre-stained protein ladder (PageRuler™, #SM0671, Fermentas International, Burlington, ON) was pipetted into the leftmost well in use. Gels were run in 1x SDS-PAGE running buffer in a Bio-Rad Protean 3 apparatus. A constant voltage of 90 V was applied for 30 minutes, to allow the proteins to migrate through the stacking gel. After this period had elapsed, the voltage was increased to 120 V for approximately one hour to facilitate protein migration.

After the protein migration was complete, the gels were removed and stacked in a "sandwich" with 0.45 µm nitrocellulose membrane (#162-0115, Bio-Rad Laboratories, Mississauga, ON) and filter paper (#2228-4657, Ahlstrom Corporation, Windsor Locks, CT). The sandwiches were then placed into a semi-dry protein transfer apparatus (Trans-Blot® SD, #170-3940, Bio-Rad Laboratories, Mississauga, ON) and run at 20 V for 45 minutes. After the transfer, the nitrocellulose membranes (blots) were removed, placed into a 5% PBST/milk solution, and allowed to block at 4°C overnight. To determine adequacy of protein loading, the transferred gels were placed into a Coomassie Brilliant Blue solution (#R250, Bio-Rad Laboratories, Mississauga, ON) and subjected to gentle agitation overnight. The next day, a Coomassie destain solution was applied and discarded until protein bands could be visualized.

After blocking overnight, a primary antibody solution was applied to the nitrocellulose blots. This normally consisted of a 1:1000 solution of primary antibody in 5% PBST/milk for PCNA, Hsp70, and HSC70, but a 1:200 dilution was used for VEGF due to low expression. The blots were probed overnight at 4°C or at room temperature for 3 hours. After probing with the primary antibody, the blots were washed three times at room temperature with 5% PBST/milk to remove excess antibody. A 1:5000 solution of secondary antibody (goat anti-mouse IgG horseradish peroxidase-conjugated, #170-6516, Bio-Rad Laboratories, Mississauga, ON) in 5% PBST/milk was then applied, and the blots were subjected to gentle agitation at room temperature for 30 minutes. The secondary antibody solution was poured off, and the blots were washed three times in 5% PBST/milk for ten minutes, two times in PBST for five minutes, and one time in PBS for five minutes.

After washing, the blots were taken to the developing darkroom. In film-safe red light, a 1:1 dilution of chemiluminescence solution (Western Lightning™ Reagent Plus, #NEL104, PerkinElmer, Inc., Waltham, MA) was added to each blot. After 1 minute of gentle agitation, the blots were gently shaken dry and placed in a film cassette. Blots were visualized on Kodak BioMax MS Film (#829-4985, Eastman Kodak Company, New Haven, CT). Exposure times were normally between 3 seconds and 10 minutes, depending on the antibody used; the VEGF blots were an exception, as these were exposed overnight. This was due to the weak signal produced by the VEGF protein in these cells.

After a satisfactory exposure was obtained, blots were stained for protein loading using Ponceau S Stain (#BP103-10, Fisher Biotech, Nepean, ON). The blots were immersed in a 0.1% w/v solution of Ponceau S in 5% v/v acetic acid on a room temperature shaker overnight. The next day, blots were destained with triple-distilled water, photocopied, and stored in plastic wrap.

However, if blots displayed excellent protein loading, they were stripped and reprobed for a different protein instead of staining with Ponceau S. The ReBlot™ Western Blot Recycling Kit (#2060, Millipore Ltd., Billerica, MA) was used to strip blots, which were then allowed to block overnight in 5% PBST/milk solution before primary antibody was applied. Subsequent steps were the same as those described above.

3.11 Data analysis and statistics

After each experiment, data were imported or typed into a Microsoft Excel® spreadsheet (Microsoft Corporation, Mississauga, Ontario). For experiments with multiwell plates, a user-defined macro was used to organize the data by nitrate treatment concentration.

Data were pooled by first averaging all readings from the same treatment group in each replicate, then averaging all replicate experiments. Pooled data were graphed using Microsoft Excel®, and reported as ± standard error of the mean.

All statistical analyses were performed with SPSS 14.0 for Windows® (SPSS Inc., Chicago, IL). Data sets were analyzed to determine mean, standard deviation, and standard error of the mean. The means of each treatment group were compared using a one-way ANOVA. Significance compared to control group was determined by two-way Dunnett's t-test post-hoc analysis, with a level of significance of $p<0.05$.

CHAPTER 4: RESULTS

4.1 Nitrate experimental assays

4.1.1 Nitrate and cell viability (Neutral Red) results

Cell cultures were processed using the Neutral Red assay protocol (see section 3.5). A visual examination of the cell cultures was also performed before the Neutral Red assay was begun. Living (viable) cells were adherent to the culture flask and exhibited normal epithelial morphology. Dead (nonviable) cells detached from the culture flask, were suspended in the culture medium, and displayed a roughly spherical or rounded morphology.

A solvent blank was read in the spectrophotometer, and an automatic blank correction was applied. Raw absorbances were read and recorded, then transferred into a Microsoft Excel® spreadsheet (Microsoft Corporation, Mississauga, Ontario). Absorbances were expressed as a percentage of the absorbance recorded for the control sample, using the following formula:

$$\%\ control\ absorbance = \frac{sample\ absorbance}{control\ absorbance} \times 100 \qquad (4.1)$$

"Viability" in the Neutral Red assay was considered the measure of the percentage of control absorbance; a high percentage denoted a high viability, while a low percentage denoted low viability. Because each combination of nitrate salt, exposure time, and tissue type was repeated three times, the data from each replicate were pooled (by

averaging) to form one graph. Each treatment group was compared to its respective control group to determine significant changes in viability.

Data from HepG2 and HEK293 trials were graphed together, in order to demonstrate possible differences in tissue specificity or susceptibility to nitrate toxicity. Combined time-point (i.e. 24, 48, and 72 hour) graphs were also constructed for HepG2 experiments to permit examination of the change in cell viability over time. Data were graphed as plus/minus standard error of the mean (SEM). Statistics were performed using SPSS 14.0 for Windows® (SPSS Inc., Chicago, IL); each data set was analysed with one-way ANOVA and a two-way Dunnett's post-hoc analysis. Values were considered significantly different from control absorbance if the resulting p-value was less than 0.05.

In order to better compare the effects of different nitrate salts, exposure times, and tissue types, an EC_{50} value was calculated from each graph of pooled data. The EC_{50} was defined as the nitrate concentration required to evoke 50% of control viability. The best-fit trendline equation for each graph was used to find the nitrate concentration that would produce 50% of control viability in each combination of nitrate salt, cell type, and exposure time. These values may be found in Table 4.1.

Table 4.1: Calculated EC_{50} values for nitrate salt Neutral Red assays in HepG2 and HEK293 cell lines. The EC_{50} values were calculated from the equation of the best-fit trendline of each graph of pooled data. The calculated EC_{50} values, expressed in mg/L of nitrate salt, were then converted to molar concentrations (mol/L). The EC_{50} was defined here as the nitrate concentration required to evoke 50% of the control absorbance in the Neutral Red assay.

Exposure time & nitrate salt	**Cell type**	**Calculated EC_{50} (mg/L)**	**Calculated EC_{50} (mol/L)**	**Comments**
24h KNO_3	HepG2	5852 mg/L*	57.9mM*	See note below
	HEK293	N/A	N/A	Upward-sloping graph; EC_{50} calculation impossible
24h NH_4NO_3	HepG2	3010 mg/L	37.6mM	
	HEK293	1807 mg/L	22.6mM	
48h KNO_3	HepG2	3862 mg/L	38.2mM	
48h NH_4NO_3	HepG2	1686 mg/L	21.1mM	
72h KNO_3	HepG2	3314 mg/L	32.8mM	
	HEK293	Unable to accurately determine EC_{50} from available data	Unable to accurately determine EC_{50} from available data	Small slope results in potentially large EC_{50} value; trendline poorly fits dataset
72h NH_4NO_3	HepG2	1744 mg/L	21.8mM	
	HEK293	1557 mg/L	19.5mM	

*Note: this EC_{50} value exceeds the range of tested concentrations and may not be accurate. It was derived from the equation of the best-fit trendline and is presented for comparison purposes only.

4.1.1.1 24 hour potassium nitrate exposure

As seen in Figure 4.1, the responses of HepG2 and HEK293 cells to increasing concentrations of nitrate were divergent. HepG2 cells experienced a brief initial increase in viability at low nitrate concentrations, with an eventual decline at the highest concentrations tested. However, the HEK293 cells experienced a linear increase in viability with increasing concentrations of nitrate. Analysis with one-way analysis of variance (one-way ANOVA) and Dunnett's post-hoc analysis revealed viability significantly different ($p<0.05$) than the control group at several nitrate exposure concentrations. The HepG2 cell cultures did not experience significantly different viability compared to the control group at any of the treatment concentrations. The HEK293 cultures experienced significantly higher viability at KNO_3 concentrations equal to and greater than 3000 mg/L. Calculation of an EC_{50} was not possible for the HEK293 exposure graph, but the approximate EC_{50} for HepG2 cells exposed to potassium nitrate for 24 hours was 5852 mg/L KNO_3 (see Table 4.1).

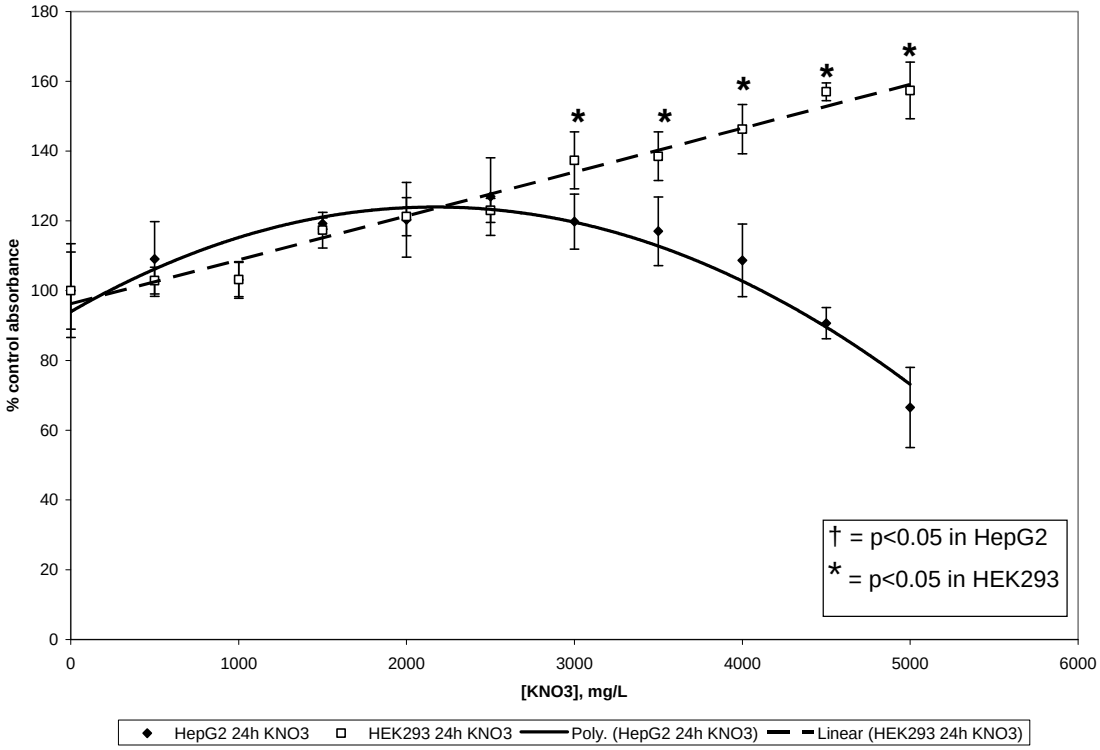


Figure 4.1: Percentage of control absorbance at 540nm in HepG2 and HEK293 cells as a function of potassium nitrate concentration in a 24 hour exposure (Neutral Red assay). Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). A closed diamond (◆) with a solid trendline was used to signify HepG2 data, while an open square (□) with a dashed trendline was used to signify HEK293 data. Significant ($p<0.05$) differences from control absorbance are denoted by the † symbol for HepG2 cells and the * symbol for HEK293 cells. No significant differences from control absorbance were observed in HepG2 cells. Significant differences from control absorbance were observed in HEK293 cells at the following concentrations: 3000 mg/L ($p=0.007$), 3500 mg/L ($p=0.005$), 4000 mg/L ($p=0.001$), 4500 mg/L ($p=0.00006$), and 5000 mg/L ($p=0.00006$).

4.1.1.2 24 hour ammonium nitrate exposure

In the 24 hour ammonium nitrate exposure, the HepG2 and HEK293 cell lines responded in a somewhat similar manner, as seen in Figure 4.2. Both cell types experienced a quadratic polynomial decline in viability when exposed to increasing concentrations of nitrate. The HepG2 cells experienced significant differences compared to control group viability at concentrations at and above 3000 mg/L NH_4NO_3. The HEK293 cells experienced significantly different viability compared to the control group at and above 1500 mg/L NH_4NO_3. Approximate EC_{50} values for 24 hour NH_4NO_3 exposure were 3010 mg/L (HepG2) and 1807 mg/L (HEK293). Because of difficulties encountered when analyzing the 24 hour HepG2 NH_4NO_3 assays, only two replicates were used to compose the HepG2 dataset.

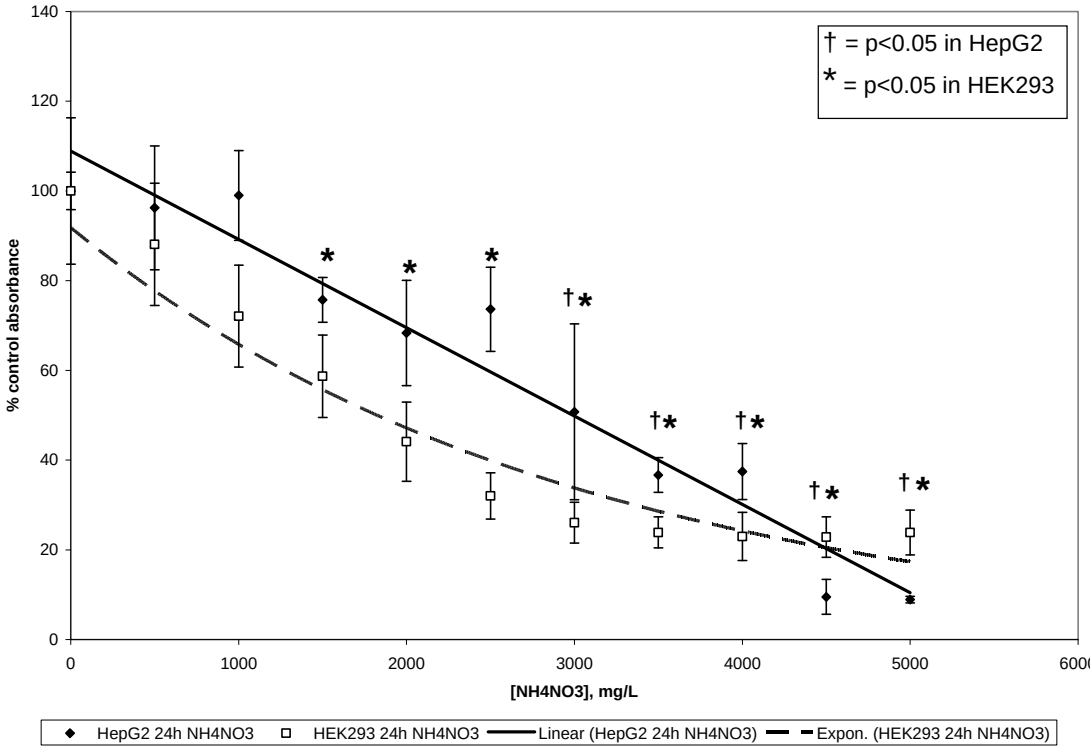


Figure 4.2: Percentage of control absorbance at 540nm in HepG2 and HEK293 cells as a function of ammonium nitrate concentration in a 24 hour exposure (Neutral Red assay). Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). A closed diamond (◆) with a solid trendline was used to signify HepG2 data, while an open square (□) with a dashed trendline was used to signify HEK293 data. Significant ($p<0.05$) differences from control absorbance are denoted by the † symbol for HepG2 cells and the * symbol for HEK293 cells. Significant differences from control absorbance were observed in HepG2 cells at the following concentrations: 3000 mg/L ($p=0.026$), 3500 mg/L ($p=0.005$), 4000 mg/L ($p=0.005$), 4500 mg/L ($p=0.0003$), and 5000 mg/L ($p=0.0003$). Significant differences from control absorbance were observed in HEK293 cells at the following concentrations: 1500 mg/L ($p=0.025$), 2000 mg/L ($p=0.002$), 2500 mg/L ($p=0.0002$), 3000 mg/L ($p=0.0001$), 3500 mg/L ($p=0.00004$), 4000 mg/L ($p=0.00003$), 4500 mg/L ($p=0.00003$), and 5000 mg/L ($p=0.00004$).

4.1.1.3 48 hour potassium nitrate exposure (HepG2 cells only)

In order to better characterize the viability changes evoked by nitrate in HepG2 cells, a 48 hour potassium nitrate exposure set was performed. These results may be seen in Figure 4.3. Instead of the parabolic trend seen in the 24 hour KNO_3 assay, this exposure demonstrated a quadratic polynomial decline in viability with increasing concentrations of potassium nitrate. Significant differences compared to control group viability were seen at 4000 mg/L, 4500 mg/L, and 5000 mg/L KNO_3. As seen in Table 4.1, the approximate EC_{50} value for 48 hour KNO_3 exposure was 3862 mg/L.

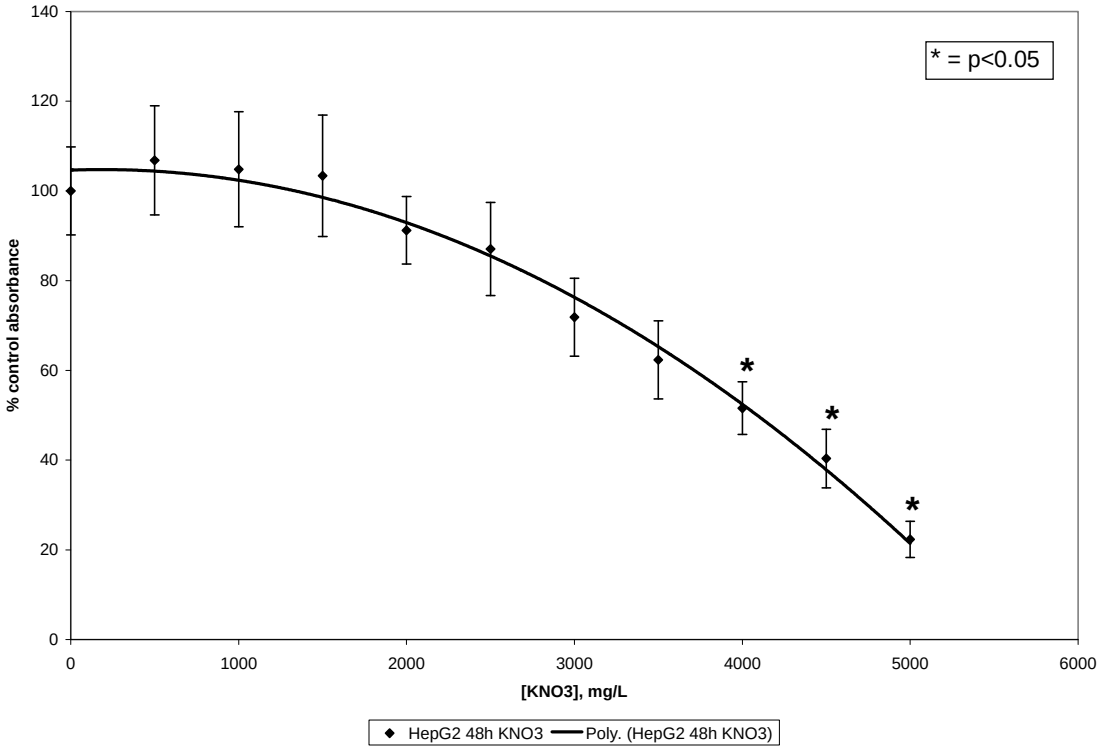


Figure 4.3: Percentage of control absorbance at 540nm in HepG2 cells as a function of potassium nitrate concentration in a 48 hour exposure (Neutral Red assay). The HEK293 cell line was not tested in this assay. Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). Significant ($p<0.05$) differences from control absorbance are denoted by the * symbol. Significant differences from control absorbance were observed in HepG2 cells at the following concentrations: 4000 mg/L ($p=0.012$), 4500 mg/L ($p=0.002$), and 5000 mg/L ($p=0.0001$).

4.1.1.4 48 hour ammonium nitrate exposure (HepG2 cells only)

A 48 hour ammonium nitrate exposure data set was performed for HepG2 cells only, in order to better understand the effect of exposure time on nitrate toxicity. These results may be seen in Figure 4.4, and show an exponential decline in cell viability with increasing concentrations of ammonium nitrate. In the 24 hour assay, the HepG2 response to NH_4NO_3 exposure followed a quadratic polynomial trend (see Figure 4.2 and section 4.1.1.2); however, this changed to a more exponential relationship in the 48 hour exposure set. Significant differences compared to control group viability were seen at concentrations at and above 2500 mg/L NH_4NO_3. The approximate EC_{50} for 48 hour ammonium nitrate exposure in HepG2 cells was 1686 mg/L.

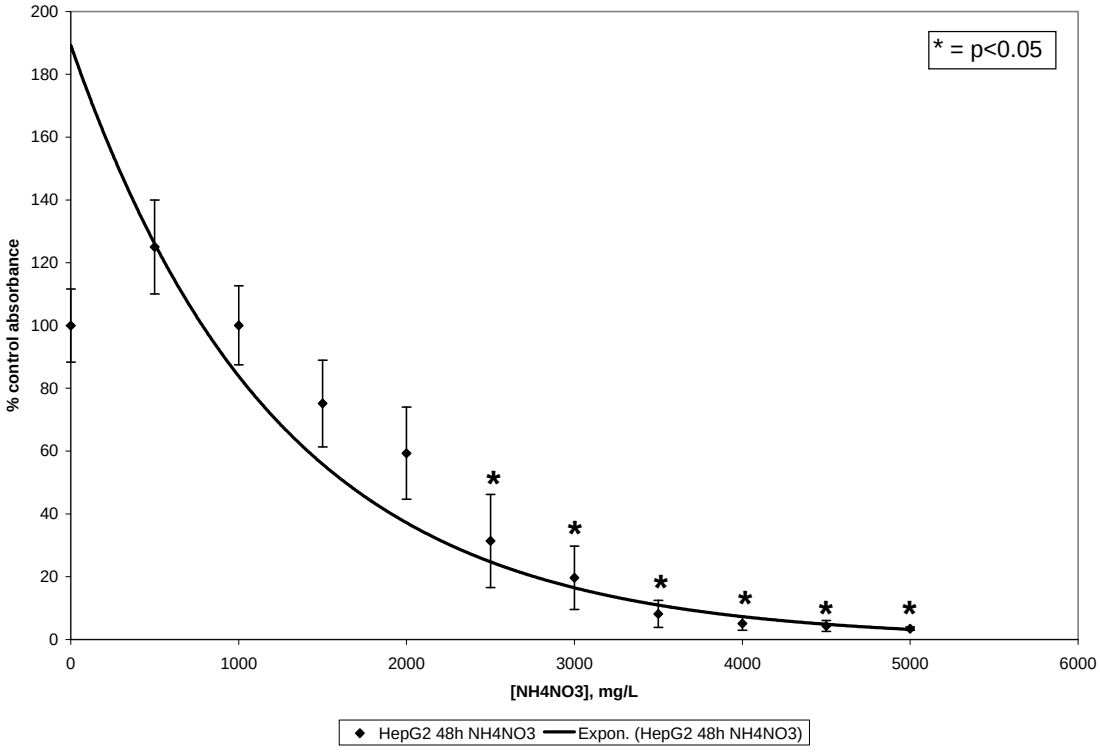


Figure 4.4: Percentage of control absorbance at 540nm in HepG2 cells as a function of ammonium nitrate concentration in a 48 hour exposure (Neutral Red assay). The HEK293 cell line was not tested in this assay. Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). Significant ($p<0.05$) differences from control absorbance are denoted by the * symbol. Significant differences from control absorbance were observed in HepG2 cells at the following concentrations: 2500 mg/L ($p=0.001$), 3000 mg/L ($p=0.0002$), 3500 mg/L ($p=0.00004$), 4000 mg/L ($p=0.00002$), 4500 mg/L ($p=0.00002$), and 5000 mg/L ($p=0.00002$).

4.1.1.5 72 hour potassium nitrate exposure

Like the 24 hour potassium nitrate exposure, the HepG2 and HEK293 cells responded quite differently to the 72 hour potassium nitrate exposure, as seen in Figure 4.5. The HepG2 cells experienced a cubic polynomial decline in viability, with significantly different viability from the control group at and above 3000 mg/L KNO_3. However, the HEK293 cells did not experience any significant decline in cell viability throughout the concentration ranges. A slight linear decline in viability was present, but no significant differences compared to control group viability were found. This was not an unexpected finding, as HEK293 cell viability appeared to increase with increasing potassium nitrate concentration in a 24 hour exposure (see section 4.1.1.1 and Figure 4.1). Based on the values calculated from the graphs, the EC_{50} for HepG2 cells was 3314 mg/L, while for HEK293 cells it was not calculable with these experimental data, owing to the seemingly minor effect of nitrate exposure on HEK293 cell viability.

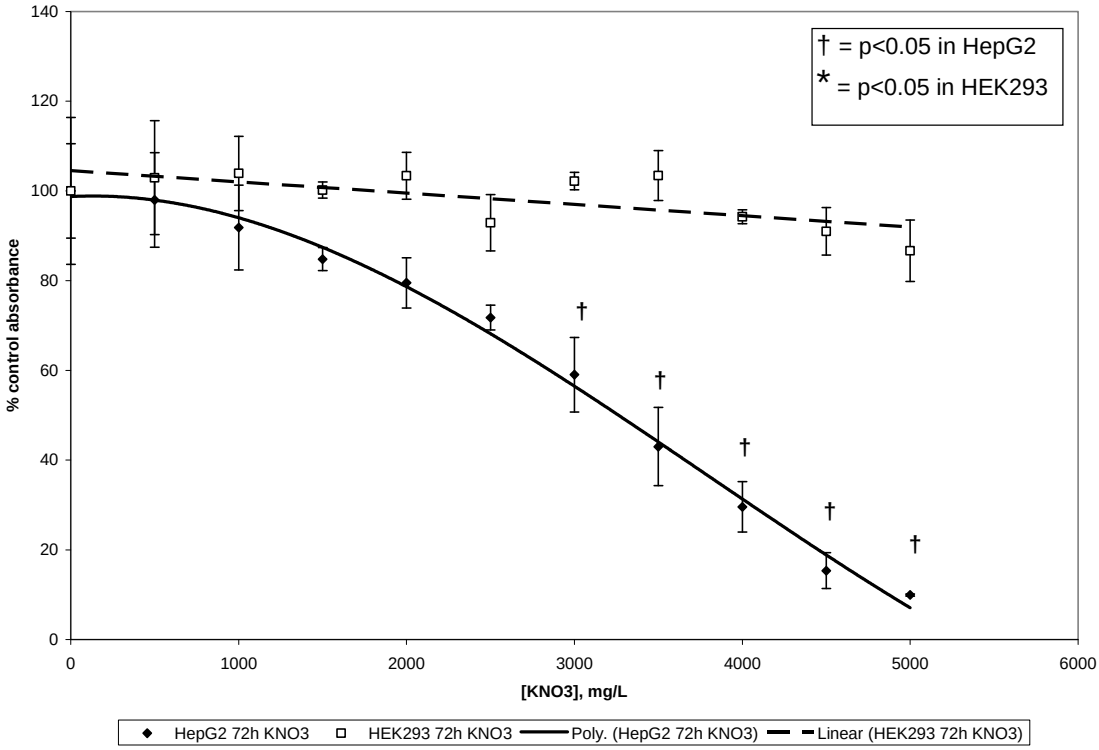


Figure 4.5: Percentage of control absorbance at 540nm in HepG2 and HEK293 cells as a function of potassium nitrate concentration in a 72 hour exposure (Neutral Red assay). Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). A closed diamond (◆) with a solid trendline was used to signify HepG2 data, while an open square (□) with a dashed trendline was used to signify HEK293 data. Significant ($p<0.05$) differences from control absorbance are denoted by the † symbol for HepG2 cells and the * symbol for HEK293 cells. Significant differences from control absorbance were observed in HepG2 cells at the following concentrations: 3000 mg/L ($p=0.004$), 3500 mg/L ($p=0.0001$), 4000 mg/L ($p=0.000004$), 4500 mg/L ($p=0.0000002$), and 5000 mg/L ($p=0.0000001$). No significant differences from control absorbance were observed in HEK293 cells.

4.1.1.6 72 hour ammonium nitrate exposure

The 72 hour ammonium nitrate exposure had a strong negative effect on cell viability in both HepG2 and HEK293 cell lines, as seen in Figure 4.6. Both cell types experienced a quadratic polynomial decline in viability with increasing NH_4NO_3 concentrations. Significant effects compared to the control group were seen at 3000 mg/L NH_4NO_3 and above in HepG2 cells, while HEK293 cells experienced significantly reduced viability at 500 mg/L NH_4NO_3 and above. Viability was reduced to below 10% of control in both cell types at concentrations greater than 4000 mg/L NH_4NO_3. Approximate EC_{50} values for the 72 hour ammonium nitrate exposure were 1744 mg/L for HepG2 cells, and 1557 mg/L for HEK293 cells.

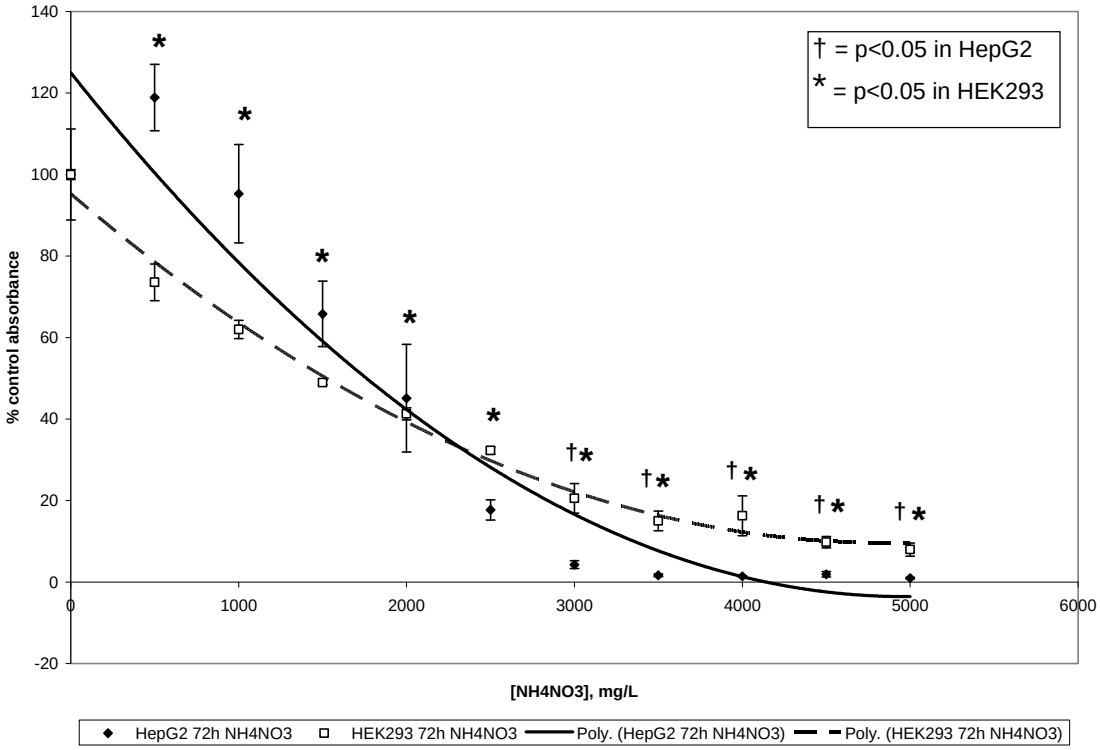


Figure 4.6: Percentage of control absorbance at 540nm in HepG2 and HEK293 cells as a function of ammonium nitrate concentration in a 72 hour exposure (Neutral Red assay). Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). A closed diamond (◆) with a solid trendline was used to signify HepG2 data, while an open square (□) with a dashed trendline was used to signify HEK293 data. Significant ($p<0.05$) differences from control absorbance are denoted by the † symbol for HepG2 cells and the * symbol for HEK293 cells. Significant differences from control absorbance were observed in HepG2 cells at the following concentrations: 3000 mg/L ($p=0.022$), 3500 mg/L ($p=0.008$), 4000 mg/L ($p=0.009$), 4500 mg/L ($p=0.002$), and 5000 mg/L ($p=0.003$). Significant differences from control absorbance were observed in HEK293 cells at the following concentrations: 500 mg/L ($p=0.000004$), 1000 mg/L ($p=0.00000001$), 1500 mg/L ($p=2.1\times10^{-9}$), 2000 mg/L ($p=2.1\times10^{-9}$), 2500 mg/L ($p=2.1\times10^{-9}$), 3000 mg/L ($p=2.1\times10^{-9}$), 3500 mg/L ($p=2.1\times10^{-9}$), 4000 mg/L ($p=2.1\times10^{-9}$), 4500 mg/L ($p=2.1\times10^{-9}$), and 5000 mg/L ($p=2.1\times10^{-9}$).

4.1.1.7 Time course for potassium nitrate exposure in HepG2 cells

In order to better determine the effect of nitrate exposure over time, data for 24, 48, and 72 hour potassium nitrate exposures in HepG2 cells were graphed together in Figure 4.7. Upon examination of the graph, it is evident that the trends in cell viability changed with increasing exposure time. The general trendline for the 24 hour exposure was parabolic, but the trendlines for the 48 and 72 hour exposures were quadratic and cubic polynomial, respectively, and showed steeper slopes with increased exposure time. As described in section 4.1.1.1, no significant differences compared to control group viability were seen in the 24 hour potassium nitrate group. However, both 48- and 72 hour groups showed significantly different viability compared to their respective control groups at 4000 mg/L KNO_3 and above. The 72 hour KNO_3 group also showed significantly reduced viability at 3000 mg/L and 3500 mg/L KNO_3. Approximate EC_{50} values for each time point were 5852 mg/L (24h KNO_3), 3862 mg/L (48h KNO_3), and 3314 mg/L (72h KNO_3).

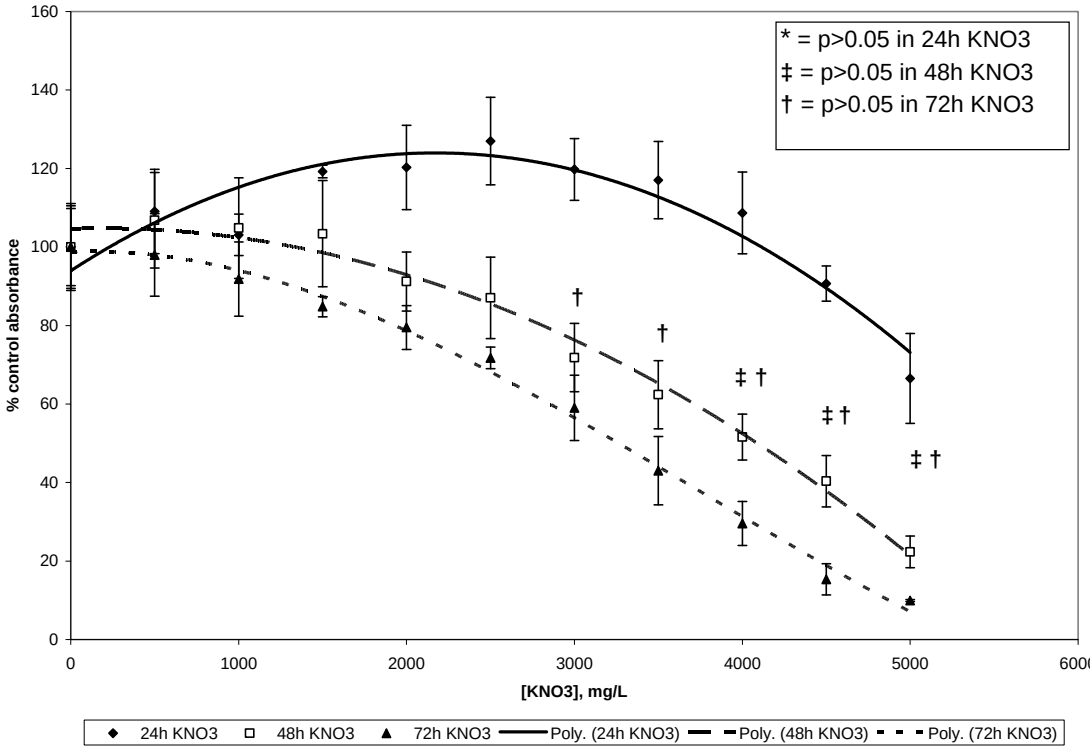


Figure 4.7: Percentage of control absorbance at 540nm in HepG2 cells as a function of potassium nitrate concentration in a 24, 48, or 72 hour exposure (Neutral Red assay). The HEK293 cell line was not tested in this assay. Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). A closed diamond (◆) with a solid trendline was used to signify 24h HepG2 data, an open square (□) with a dashed trendline was used to signify 48h HepG2 data, and a closed triangle (▲) with a dotted trendline was used to signify 72h HepG2 data. Significant ($p<0.05$) differences from control absorbance are denoted by the * symbol for the HepG2 24h KNO_3 exposure, the ‡ symbol for the HepG2 48h KNO_3 exposure, and the † symbol for the HepG2 72h KNO_3 exposure. No significant differences from control absorbance were observed in the HepG2 cells exposed to potassium nitrate for 24 hours. Significant differences from control absorbance were observed in HepG2 cells exposed to KNO_3 for 48 hours at the following concentrations: 4000 mg/L ($p=0.012$), 4500 mg/L ($p=0.002$), and 5000 mg/L ($p=0.0001$). Significant differences from control absorbance were observed in HepG2 cells exposed to KNO_3 for 72 hours at the following concentrations: 3000 mg/L ($p=0.004$), 3500 mg/L ($p=0.0001$), 4000 mg/L ($p=0.000004$), 4500 mg/L ($p=0.0000002$), and 5000 mg/L ($p=0.0000001$).

4.1.1.8 Time course for ammonium nitrate exposure in HepG2 cells

The data from 24-, 48-, and 72 hour ammonium nitrate exposures in HepG2 cells were graphed together to better illustrate the effects of nitrate exposure over time. These data may be seen in Figure 4.8. There was a noticeable difference between the trendlines for the 24 hour exposure and the 72 hour exposure, which were quadratic polynomial, and the trendline for the 48 hour exposure period, which was exponential. Significant differences to their respective control group viability were seen in all time points at and above 3000 mg/L NH_4NO_3. The 48 hour NH_4NO_3 exposure group also had significantly different viability compared to its control group at 2500 mg/L NH_4NO_3. Approximate EC_{50} values for each time point were 3010 mg/L (24h NH_4NO_3), 1686 mg/L (48h NH_4NO_3), and 1744 mg/L (72h NH_4NO_3).

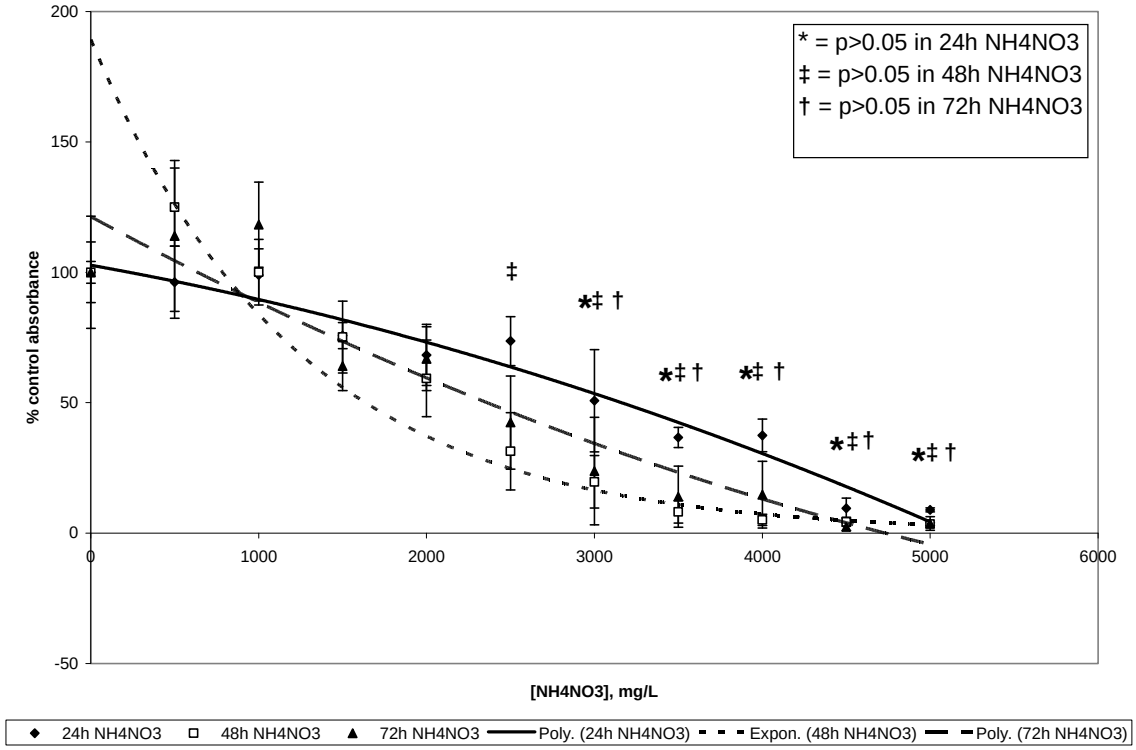


Figure 4.8: Percentage of control absorbance at 540nm in HepG2 cells as a function of ammonium nitrate concentration in a 24, 48, or 72 hour exposure (Neutral Red assay). The HEK293 cell line was not tested in this assay. Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). A closed diamond (◆) with a solid trendline was used to signify 24h HepG2 data, an open square (□) with a dashed trendline was used to signify 48h HepG2 data, and a closed triangle (▲) with a dotted trendline was used to signify 72h HepG2 data. Significant ($p<0.05$) differences from control absorbance are denoted by the * symbol for the HepG2 24h NH_4NO_3 exposure, the ‡ symbol for the HepG2 48h NH_4NO_3 exposure, and the † symbol for the HepG2 72h NH_4NO_3 exposure. Significant differences from control absorbance were observed in the HepG2 cells exposed to NH_4NO_3 for 24 hours at the following concentrations: 3000 mg/L (p=0.026), 3500 mg/L (p=0.005), 4000 mg/L (p=0.005), 4500 mg/L (p=0.0003), and 5000 mg/L (p=0.0003). Significant differences from control absorbance were observed in HepG2 cells exposed to NH_4NO_3 for 48 hours at the following concentrations: 2500 mg/L (p=0.001), 3000 mg/L (p=0.0002), 3500 mg/L (p=0.00004), 4000 mg/L (p=0.00002), 4500 mg/L (p=0.00002), and 5000 mg/L (p=0.00002). Significant differences from control absorbance were observed in HepG2 cells exposed to NH_4NO_3 for 72 hours at the following concentrations: 3000 mg/L (p=0.022), 3500 mg/L (p=0.008), 4000 mg/L (p=0.009), 4500 mg/L (p=0.002), and 5000 mg/L (p=0.003).

4.1.1.9 Time course for potassium nitrate exposure in HEK293 cells

To determine the effect of exposure time on HEK293 cell viability after exposure to potassium nitrate, the data from the 24- and 72 hour exposures were graphed together in Figure 4.9. A clear difference is visible between the two trendlines; the 24 hour dataset shows a steady linear increase in viability, while the 72 hour dataset shows a slight linear decline in viability with increasing KNO_3 concentration. Significantly increased viability in HEK293 cells exposed to KNO_3 for 24 hours was observed at concentrations at and above 3000 mg/L KNO_3. No significant differences in viability compared to the control group were observed in the 72 hour KNO_3 dataset. A EC_{50} was not available for the 24 hour data due to the increase in viability observed. The EC_{50} value for the 72 hour KNO_3 HEK293 exposure was not calculable based on the data obtained.

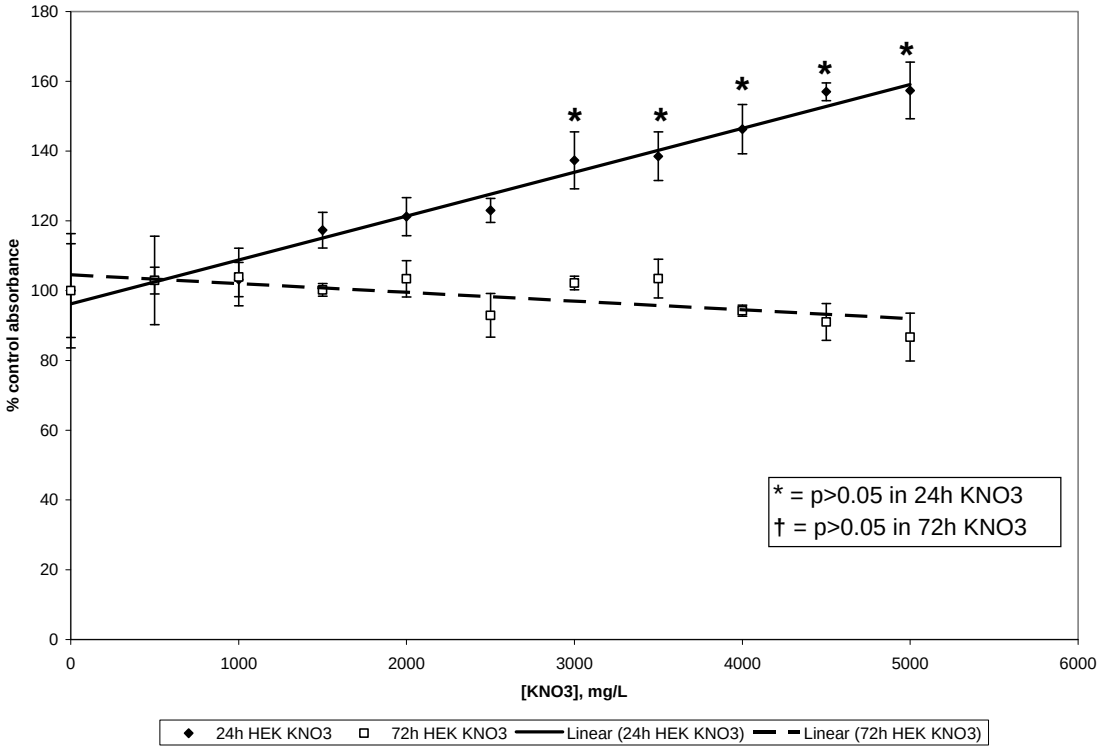


Figure 4.9: Percentage of control absorbance at 540nm in HEK293 cells as a function of potassium nitrate concentration in a 24 or 72 hour exposure (Neutral Red assay). The HepG2 cell line was not tested in this assay. Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). A closed diamond (◆) with a solid trendline was used to signify 24h HEK293 data, and an open square (□) with a dashed trendline was used to signify 72h HEK293 data. Significant ($p<0.05$) differences from control absorbance are denoted by the * symbol for the HEK293 24h KNO_3 exposure, and the † symbol for the HEK293 72h KNO_3 exposure. Significant differences from control absorbance were observed in the HEK293 cells exposed to KNO_3 for 24 hours at the following concentrations: 3000 mg/L ($p=0.007$), 3500 mg/L ($p=0.005$), 4000 mg/L ($p=0.001$), 4500 mg/L ($p=0.00006$), and 5000 mg/L ($p=0.00006$). No significant differences from control absorbance were observed in the HEK293 cells exposed to potassium nitrate for 72 hours.

4.1.1.10 Time course for ammonium nitrate exposure in HEK293 cells

To determine the effect of ammonium nitrate exposure on HEK293 cells over time, the 24 and 72 hour HEK293 NH_4NO_3 exposure datasets were graphed together in Figure 4.10. Both trendlines show a similar quadratic polynomial decline in cell viability with increasing NH_4NO_3 concentration, with the 72 hour dataset showing a greater decrease in cell viability at higher NH_4NO_3 concentrations. Significantly decreased viability compared to the control group was noted for both exposure groups at concentrations above and including 1500 mg/L NH_4NO_3. Additional significantly decreased viability for the 72 hour exposure group was noted at 500 mg/L and 1000 mg/L NH_4NO_3. Approximate EC_{50} values for each time point were 1807 mg/L (24h NH_4NO_3) and 1557 mg/L (72h NH_4NO_3).

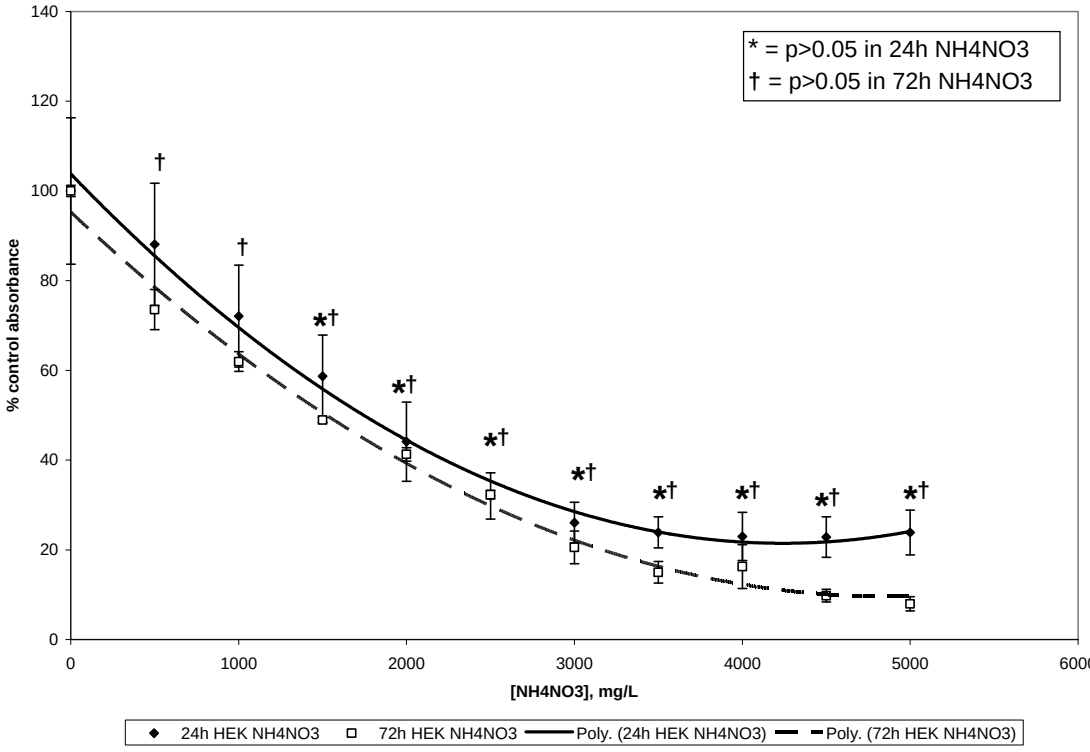


Figure 4.10: Percentage of control absorbance at 540nm in HEK293 cells as a function of ammonium nitrate concentration in a 24 or 72 hour exposure (Neutral Red assay). The HepG2 cell line was not tested in this assay. Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). A closed diamond (◆) with a solid trendline was used to signify 24h HEK293 data, and an open square (□) with a dashed trendline was used to signify 72h HEK293 data. Significant ($p<0.05$) differences from control absorbance are denoted by the * symbol for the HEK293 24h NH_4NO_3 exposure, and the † symbol for the HEK293 72h NH_4NO_3 exposure. Significant differences from control absorbance were observed in the HEK293 cells exposed to NH_4NO_3 for 24 hours at the following concentrations: 1500 mg/L ($p=0.025$), 2000 mg/L ($p=0.002$), 2500 mg/L ($p=0.0002$), 3000 mg/L ($p=0.0001$), 3500 mg/L ($p=0.00004$), 4000 mg/L ($p=0.00003$), 4500 mg/L ($p=0.00003$), and 5000 mg/L ($p=0.00004$). Significant differences from control absorbance were observed in the HEK293 cells exposed to NH_4NO_3 for 72 hours at the following concentrations: 500 mg/L ($p=0.000004$), 1000 mg/L ($p=0.00000001$), 1500 mg/L ($p=2.1\times10^{-9}$), 2000 mg/L ($p=2.1\times10^{-9}$), 2500 mg/L ($p=2.1\times10^{-9}$), 3000 mg/L ($p=2.1\times10^{-9}$), 3500 mg/L ($p=2.1\times10^{-9}$), 4000 mg/L ($p=2.1\times10^{-9}$), 4500 mg/L ($p=2.1\times10^{-9}$), and 5000 mg/L ($p=2.1\times10^{-9}$).

4.1.2 Nitrate and cell proliferation (BrdU ELISA) results

Both HepG2 and HEK293 cells were used in proliferation assays, and were processed using the 5-bromo-2'-deoxyuridine enzyme-linked immunosorbent assay (BrdU ELISA) protocol as described in section 3.6. Raw absorbances were imported from KC4 Kineticalc for Windows software version 3.3 (Bio-Tek Instruments Inc., Winooski, VT) into a Microsoft Excel® (Microsoft Corporation, Mississauga, Ontario) spreadsheet. A user-defined macro was used to sort absorbance values into their corresponding treatment concentrations. Because each treatment was replicated in six wells per experimental plate, the mean of the absorbances from each treatment group was reported and graphed. Any obvious artifact values, such as those where experimental error was known to occur, or those more than two standard deviations above or below the calculated mean for the data, were discarded. Absorbances were expressed as a percentage of the absorbance recorded for the control sample, using the following formula:

$$\%\, control\ absorbance = \frac{sample\ absorbance}{control\ absorbance} \times 100 \qquad (4.1)$$

The degree of proliferation in the BrdU assay was considered the measure of the percentage of control absorbance; a high percentage denoted high proliferation, while a low percentage denoted low proliferation.

Each combination of cell type, nitrate salt, and exposure time was repeated three times; however, due to variations between each replicate only one representative trace is displayed here. The majority of experimental graphs appeared similar, yet were "shifted" up or down on the y-axis relative to each other. Methods to normalize the data were not

readily discernible, so one graph was chosen to be the representative trace from each set of experimental data. Attempts to pool the data from each replicate resulted in unacceptably high error and loss of statistical significance.

Data from HepG2 and HEK293 trials were graphed together, in order to demonstrate possible differences in tissue specificity or susceptibility to nitrate toxicity. Data were graphed as plus/minus standard error of the mean (SEM). Statistics were performed using SPSS 14.0 for Windows® (SPSS Inc., Chicago, IL); each data set was analysed with one-way ANOVA and a two-way Dunnett's post-hoc analysis. Values were considered significantly different from control absorbance if the resulting p-value was less than 0.05.

In order to better compare the effects of different nitrate salts, exposure times, and tissue types, an EC_{50} value was calculated from each graph of representative data. The EC_{50} was defined as the nitrate concentration required to evoke 50% of control proliferation. The best-fit trendline equation for each graph was used to find the nitrate concentration that would produce 50% of control proliferation in each combination of nitrate salt, cell type, and exposure time. These values may be found in Table 4.2. Several trendlines, including exponential, logarithmic, linear, and quadratic polynomial, were examined to see which was the best fit for the data. However, some datasets did not conform closely to a particular trendline; in this case, the best-fitting trendline was used, and a notation was made on the EC_{50} table.

Table 4.2: Calculated EC_{50} values for nitrate salt BrdU ELISA assays in HepG2 and HEK293 cell lines. The EC_{50} values were calculated from the equation of the best-fit trendline of each graph of representative data. The calculated EC_{50} values, expressed in mg/L of nitrate salt, were then converted to molar concentrations (mol/L). The EC_{50} is defined here as the nitrate concentration required to evoke 50% of the control absorbance in the BrdU ELISA assay.

Exposure time & nitrate salt	**Cell type**	**Calculated EC_{50} (mg/L)**	**Calculated EC_{50} (mol/L)**	**Comments**
24h KNO_3	HepG2	1943 mg/L	19.2mM	
	HEK293	1737 mg/L	17.2mM	Trendline poorly fits dataset
24h NH_4NO_3	HepG2	734 mg/L	9.17mM	
	HEK293	1442 mg/L	18.0mM	
72h KNO_3	HepG2	730 mg/L	7.22mM	Trendline poorly fits dataset
	HEK293	3631 mg/L	36.0mM	Trendline poorly fits dataset
72h NH_4NO_3	HepG2	~2.5 mg/L	31.2µM	Graph equation did not produce usable EC_{50} value; this is an estimated value (trendline poorly fits dataset)
	HEK293	471 mg/L	5.88mM	

4.1.2.1 24 hour potassium nitrate exposure

As seen in Figure 4.11, both cell types responded in a similar manner to a 24 hour potassium nitrate exposure. Both cell types experienced a decline in proliferation with increasing potassium nitrate concentrations; the HepG2 cells demonstrated a strong exponential decline, while the HEK293 cells demonstrated a weaker quadratic polynomial decline. From the shape of the trendlines, it appears that the HEK293 cells were more sensitive at lower KNO_3 concentrations, while the HepG2 cells appeared to be more sensitive at higher KNO_3 concentrations. Proliferation in HepG2 cells at low KNO_3 concentrations appears to increase slightly; values for 1 µg/L, 50 µg/L, 3 mg/L, 45 mg/L, and 250 mg/L are all elevated compared to the control group, although these differences are not significant. Both cell types experienced significant ($p<0.05$) differences in proliferation compared to their respective control groups at and above 2500 mg/L KNO_3. The HepG2 cells experienced additional significantly different proliferation at 1500 mg/L, while the HEK293 cells were significantly different from control at 1000 mg/L and 2000 mg/L KNO_3. The approximate EC_{50} values for a 24 hour KNO_3 exposure were 1943 mg/L (HepG2) and 1737 mg/L (HEK293).

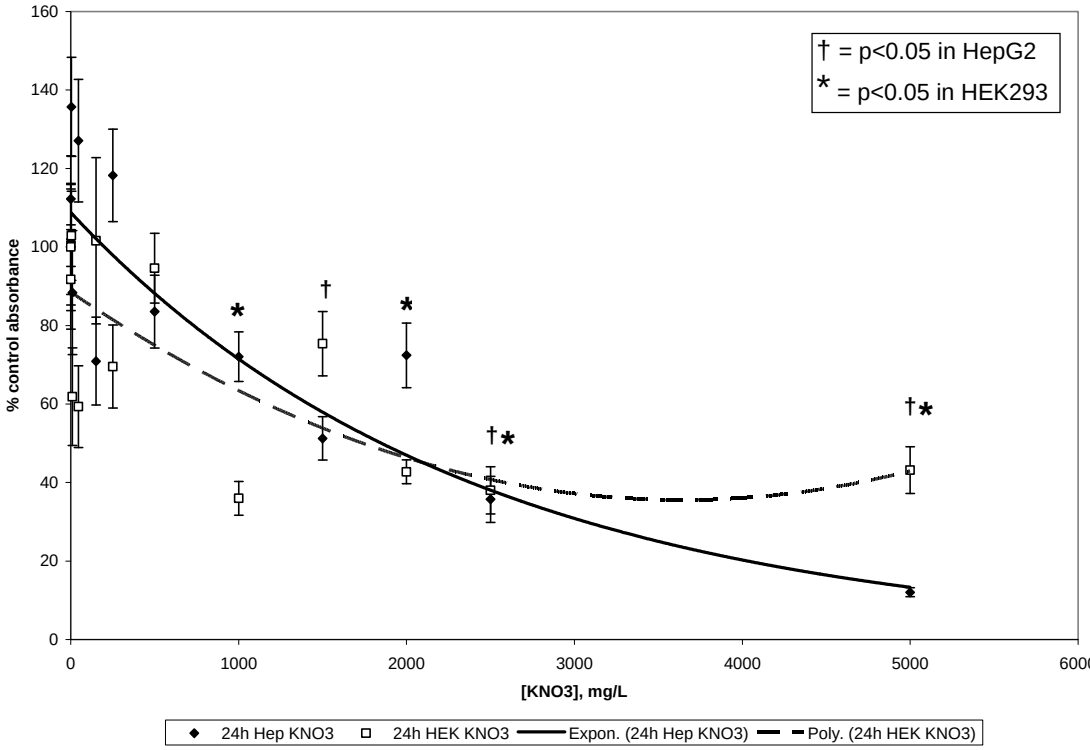


Figure 4.11: Percent of control absorbance at 450nm in HepG2 and HEK293 cells as a function of potassium nitrate concentration in a 24 hour exposure (BrdU ELISA). This graph contains representative traces from each experiment, not pooled data from several replicates. Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). A closed diamond (◆) with a solid trendline was used to signify HepG2 data, and an open square (□) with a dashed trendline was used to signify HEK293 data. Significant ($p<0.05$) differences from control absorbance are denoted by the † symbol for HepG2 cells and the * symbol for HEK293 cells. Significant differences from control absorbance were observed in the HepG2 cells exposed to KNO_3 for 24 hours at the following concentrations: 1500 mg/L ($p=0.026$), 2500 mg/L ($p=0.001$), and 5000 mg/L ($p=3.4x10^{-6}$). Significant differences from control absorbance were observed in the HEK293 cells exposed to KNO_3 for 24 hours at the following concentrations: 1000 mg/L ($p=0.001$), 2000 mg/L ($p=0.006$), 2500 mg/L ($p=0.002$), and 5000 mg/L ($p=0.006$).

4.1.2.2 24 hour ammonium nitrate exposure

The response to a 24 hour ammonium nitrate exposure in both HepG2 and HEK293 cells was very similar, as seen in Figure 4.12. Both cell types experienced an exponential decline in proliferation compared to their respective control groups. Significant ($p<0.05$) differences from control group proliferation were observed in both cell types at concentrations at and above 1500 mg/L NH_4NO_3, with additional significant points in HepG2 cells at 250 mg/L, 500 mg/L, and 1000 mg/L NH_4NO_3. As with the 24 hour potassium nitrate exposure (see section 4.1.2.1), an initial increase in proliferation compared to each control group was observed at low NH_4NO_3 concentrations in both cell types. The approximate EC_{50} values for a 24 hour NH_4NO_3 exposure were 734 mg/L (HepG2) and 1442 mg/L (HEK293).

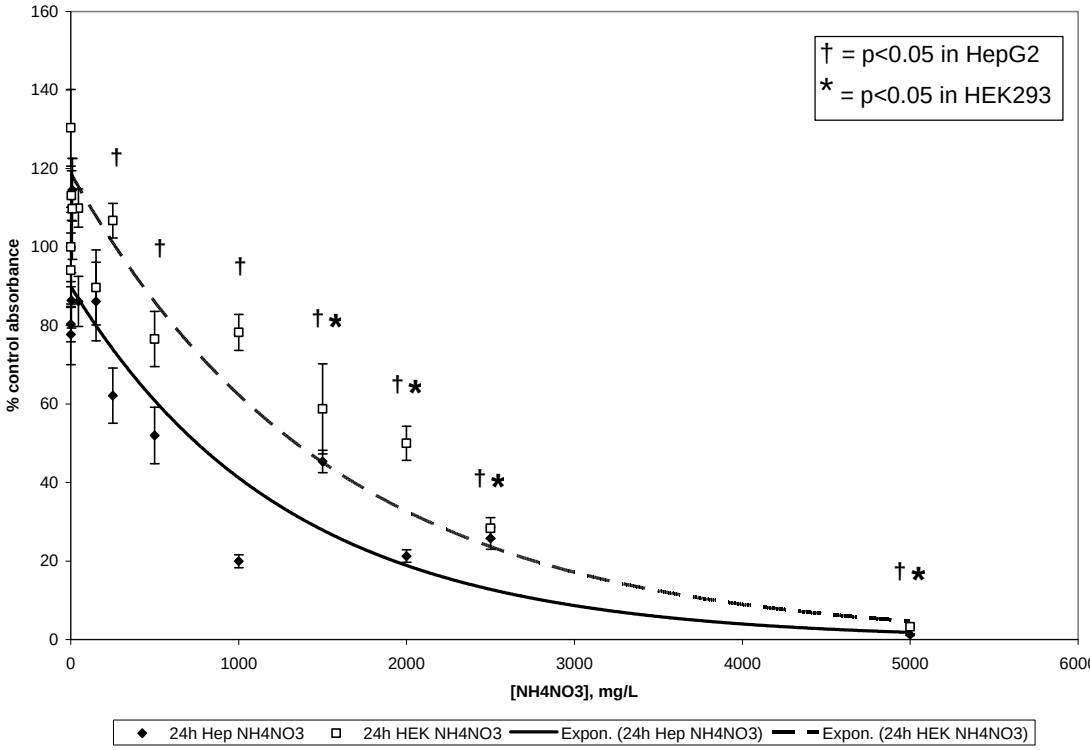


Figure 4.12: Percent of control absorbance at 450nm in HepG2 and HEK293 cells as a function of ammonium nitrate concentration in a 24 hour exposure (BrdU ELISA). This graph contains representative traces from each experiment, not pooled data from several replicates. Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). A closed diamond (◆) with a solid trendline was used to signify HepG2 data, and an open square (□) with a dashed trendline was used to signify HEK293 data. Significant ($p<0.05$) differences from control absorbance are denoted by the † symbol for HepG2 cells and the * symbol for HEK293 cells. Significant differences from control absorbance were observed in the HepG2 cells exposed to NH_4NO_3 for 24 hours at the following concentrations: 250 mg/L ($p=0.0004$), 500 mg/L ($p=4.3\times10^{-6}$), 1000 mg/L ($p=1.2\times10^{-8}$), 1500 mg/L ($p=1.8\times10^{-7}$), 2000 mg/L ($p=1.2\times10^{-8}$), 2500 mg/L ($p=1.2\times10^{-8}$), and 5000 mg/L ($p=1.2\times10^{-8}$). Significant differences from control absorbance were observed in the HEK293 cells exposed to NH_4NO_3 for 24 hours at the following concentrations: 1500 mg/L ($p=0.004$), 2000 mg/L ($p=0.0003$), 2500 mg/L ($p=1.2\times10^{-7}$), and 5000 mg/L ($p=2.1\times10^{-8}$).

4.1.2.3 72 hour potassium nitrate exposure

The general shape of the response to a 72 hour KNO_3 exposure in the two cell types was similar, as seen in Figure 4.13, but the magnitude of the responses were quite different. While both cell types showed a gradual decline in proliferation over the range of increasing KNO_3 concentrations, the HepG2 cells appeared to be more sensitive to the deleterious effects of potassium nitrate. The HEK293 cells appeared to experience a large and sustained increase in cell proliferation compared to their control group over the range of most of the KNO_3 concentrations tested, although a subtle downward trend is visible. For some data points on the HEK293 graphs, the error, represented as standard error of the mean, was large, especially in the 150 mg/L and 1500 mg/L KNO_3 test concentrations. Conversely, the HepG2 cells appeared to experience a decline in proliferation, even at low KNO_3 concentrations. At 5000 mg/L KNO_3, the HepG2 cells reached a negative percentage of control absorbance, after subtraction of the blank value. This negative value prevented the use of an exponential trendline for this trace, so a quadratic polynomial trendline was used. Significant ($p<0.05$) differences from control group proliferation were observed in HepG2 cells at and above 150 mg/L KNO_3, as well as at 3 mg/L. No significant differences in proliferation compared to the control group were found in HEK293 cells subjected to a 72 hour KNO_3 exposure. Approximate EC_{50} values for a 72 hour potassium nitrate exposure were 730 mg/L for HepG2 cells, and 3631 mg/L for HEK293 cells.

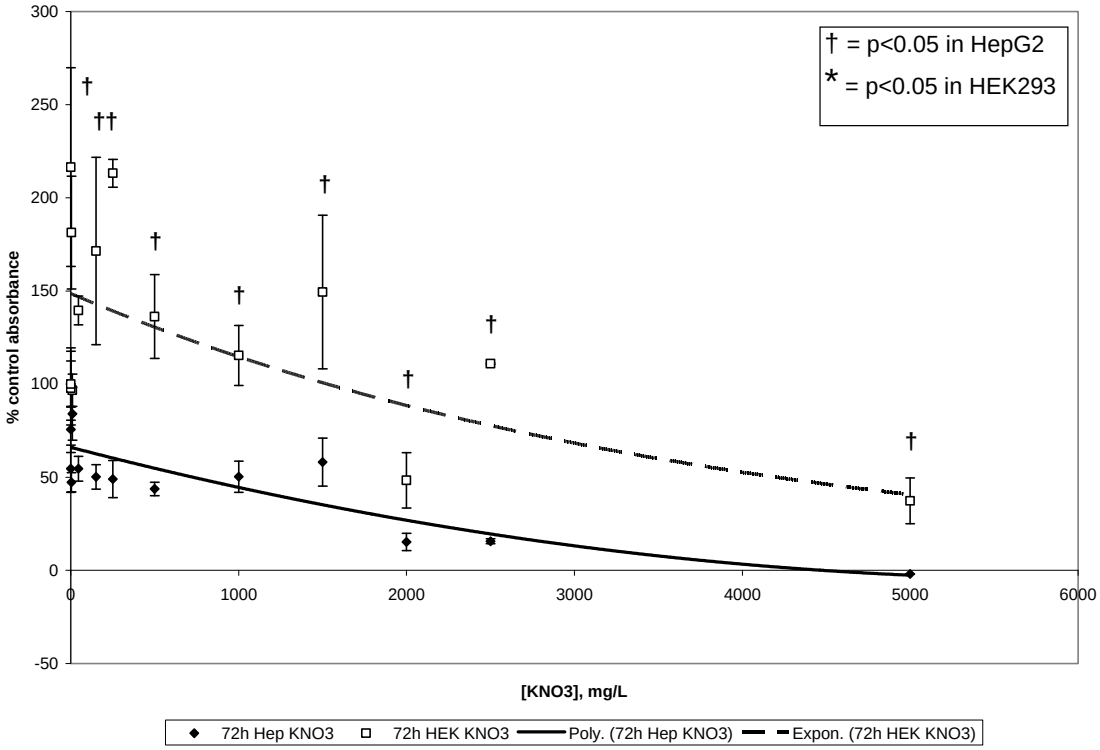


Figure 4.13: Percent of control absorbance at 450nm in HepG2 and HEK293 cells as a function of potassium nitrate concentration in a 72 hour exposure (BrdU ELISA). This graph contains representative traces from each experiment, not pooled data from several replicates. Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). A closed diamond (◆) with a solid trendline was used to signify HepG2 data, and an open square (□) with a dashed trendline was used to signify HEK293 data. Significant ($p<0.05$) differences from control absorbance are denoted by the † symbol for HepG2 cells and the * symbol for HEK293 cells. Significant differences from control absorbance were observed in the HepG2 cells exposed to KNO_3 for 72 hours at the following concentrations: 3 mg/L ($p=0.02$), 150 mg/L ($p=0.04$), 250 mg/L ($p=0.03$), 500 mg/L ($p=0.01$), 1000 mg/L ($p=0.04$), 2000 mg/L ($p=3.0\times10^{-5}$), 2500 mg/L ($p=3.3\times10^{-5}$), and 5000 mg/L ($p=4.4\times10^{-7}$). No significant differences were found compared to control absorbance in HEK293 cells exposed to KNO_3 for 72 hours.

4.1.2.4 72 hour ammonium nitrate exposure

Exposure to ammonium nitrate for 72 hours had a strong deleterious effect on cell proliferation in HepG2 and HEK293 cells, as seen in Figure 4.14. Both cell types experienced a large decline in cell proliferation, with negative proliferation values compared to their respective control groups at 5000 mg/L NH_4NO_3. The HepG2 cells also experienced negative proliferation values at values equal to and greater than 500 mg/L NH_4NO_3, as well as 50 µg/L and 150 mg/L. Due to the negative proliferation values, it was not possible to fit exponential trendlines to this data. Thus, a quadratic polynomial trendline was used. As with other BrdU ELISA assays discussed in this section, the 72 hour ammonium nitrate exposure results also demonstrated some instances of elevated proliferation at low NH_4NO_3 concentrations in both cell types. However, proliferation rapidly decreased at increasing concentrations of NH_4NO_3. No significant differences compared to control group proliferation were observed in HepG2 cells; however, this may be due to the large error observed in the HepG2 control group. Significant ($p<0.05$) differences from control group proliferation were observed in HEK293 cells at 50 µg/L, 10 mg/L, 150 mg/L, as well as at and above 500 mg/L NH_4NO_3. Obtaining an EC_{50} value for NH_4NO_3 exposure in HepG2 cells in this experiment was difficult. The best-fit trendline equation did not produce an appropriate EC_{50} value, so the determination was based on estimation. This was complicated by the large variability in data points at low concentrations, as a great deal of fluctuation in cell proliferation existed. The estimated EC_{50} value for a 72 hour ammonium nitrate exposure in HepG2 cells was ~2.5 mg/L. The approximate EC_{50} value for a 72 hour ammonium nitrate exposure in HEK293 cells was 471 mg/L.

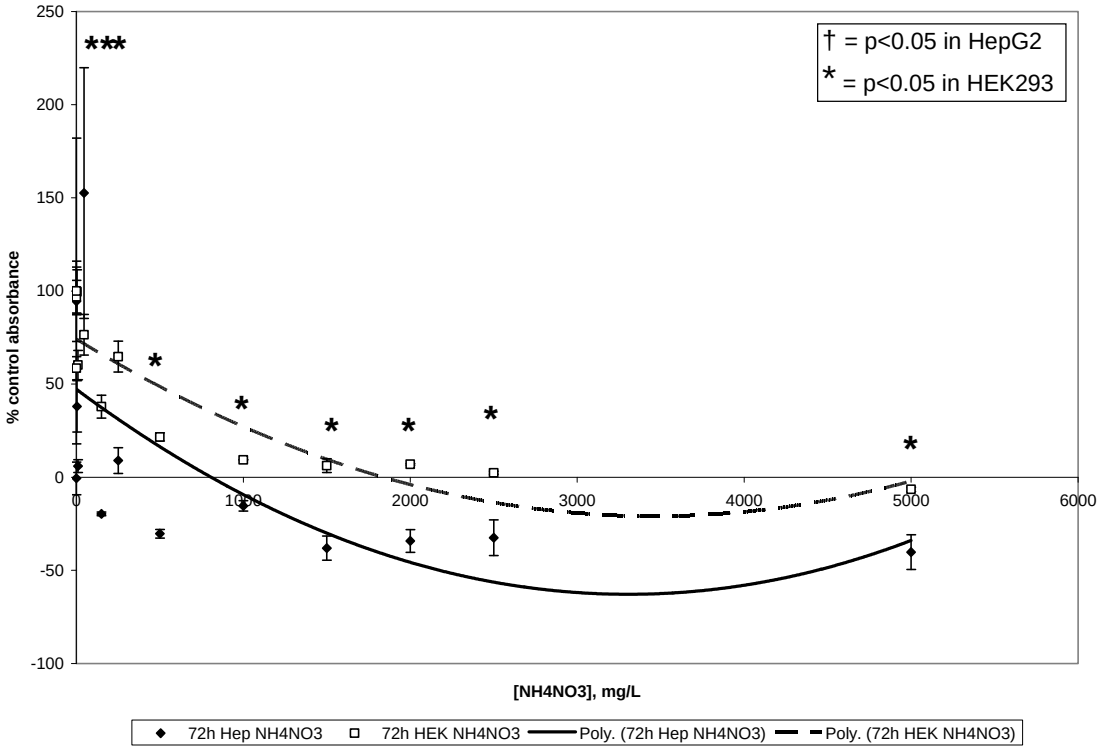


Figure 4.14: Percent of control absorbance at 450nm in HepG2 and HEK293 cells as a function of ammonium nitrate concentration in a 72 hour exposure (BrdU ELISA). This graph contains representative traces from each experiment, not pooled data from several replicates. Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). A closed diamond (◆) with a solid trendline was used to signify HepG2 data, and an open square (□) with a dashed trendline was used to signify HEK293 data. Significant ($p<0.05$) differences from control absorbance are denoted by the † symbol for HepG2 cells and the * symbol for HEK293 cells. No significant differences were found compared to control absorbance in HepG2 cells exposed to NH_4NO_3 for 72 hours. Significant differences from control absorbance were observed in the HEK293 cells exposed to NH_4NO_3 for 72 hours at the following concentrations: 50µg/L ($p=0.03$), 10 mg/L ($p=0.046$), 150 mg/L ($p=0.0003$), 500 mg/L ($p=2.6\times10^{-6}$), 1000 mg/L ($p=8.1\times10^{-8}$), 1500 mg/L ($p=4.3\times10^{-8}$), 2000 mg/L ($p=4.9\times10^{-8}$), 2500 mg/L ($p=2.8\times10^{-8}$), and 5000 mg/L ($p=2.1\times10^{-8}$).

4.2 Nitrate control and alternate salt cytotoxicity assays

In order to better determine the causes of the results observed in section 4.1.1, several control assays were designed. Assays to examine the variability of readings in the Neutral Red assay in cells exposed only to media are described in sections 4.2.1.1 and 4.2.1.2. Neutral Red assays were performed on cells exposed to different (alternate) salts of potassium, ammonium, and nitrate; see sections 4.2.1.3 – 4.2.1.5. Similarly designed experiments using the BrdU ELISA assay may be found in section 4.2.2. Table 4.3 details the approximate EC_{50} values for each Neutral Red alternate salt graph, while Table 4.4 details the EC_{50} values for the BrdU ELISA alternate salt graphs. Finally, to verify the results of the Neutral Red viability assay, the resazurin assay was performed with HepG2 and HEK293 cells exposed to ammonium nitrate for 24 hours. The EC_{50} results for the resazurin assay may be found in Table 4.5.

4.2.1 Neutral Red control and alternate salt assay results

4.2.1.1 24 hour HepG2 media control group

Ten flasks of HepG2 cells were obtained following the Neutral Red procedures described in section 3.5. These flasks were processed using the same protocol as the experimental groups; however, the cells in this assay received an aliquot of culture media instead of a nitrate treatment. After a 24 hour exposure period, the flasks were harvested using the same procedure described in section 3.5. The lysate from each flask was read twice in the spectrophotometer, to determine if variation existed between each absorbance measurement. Raw absorbances were then graphed in Figure 4.15. A one-way ANOVA with a Bonferroni post-hoc analysis was used to determine if any flasks were significantly ($p<0.05$) different from all others. Only one flask (#9) was found to

Table 4.3: Calculated EC_{50} values for alternate salt Neutral Red assays in HepG2 and HEK293 cell lines. The EC_{50} values were calculated from the equation of the best-fit trendline of each graph of pooled data. The calculated EC_{50} values, expressed in mg/L of alternate salt, were then converted to molar concentrations (mol/L). The EC_{50} is defined here as the nitrate concentration required to evoke 50% of the control absorbance in the Neutral Red assay.

Exposure time & alternate salt	**Cell type**	**Calculated EC_{50} (mg/L)**	**Calculated EC_{50} (mol/L)**	**Comments**
48h CH_3COOK	HepG2	1773 mg/L	18.1mM	
48h CH_3COONH_4	HepG2	3577 mg/L	46.4mM	
	HEK293	1328 mg/L	17.2mM	
48h $NaNO_3$	HepG2	5820 mg/L*	68.5mM*	See note below
	HEK293	Unable to accurately determine EC_{50} from available data	Unable to accurately determine EC_{50} from available data	Small slope results in potentially large EC_{50} value; trendline poorly fits dataset.

*Note: this EC_{50} value exceeds the range of tested concentrations and may not be accurate. It was derived from the equation of the best-fit trendline and is presented for comparison purposes only.

Table 4.4: Calculated EC_{50} values for alternate salt BrdU ELISA assays in HepG2 and HEK293 cell lines. The EC_{50} values were calculated from the equation of the best-fit trendline of each graph of representative data. The calculated EC_{50} values, expressed in mg/L of alternate salt, were then converted to molar concentrations (mol/L). The EC_{50} is defined here as the nitrate concentration required to evoke 50% of the control absorbance in the BrdU ELISA assay.

Exposure time & alternate salt	**Cell type**	**Calculated EC_{50} (mg/L)**	**Calculated EC_{50} (mol/L)**	**Comments**
48h CH_3COOK	HepG2	1248 mg/L	12.7mM	
48h CH_3COONH_4	HepG2	943 mg/L	12.2mM	
	HEK293	907 mg/L	11.8mM	
48h $NaNO_3$	HepG2	Unable to accurately determine EC_{50} from available data	Unable to accurately determine EC_{50} from available data	Trendline poorly fits dataset
	HEK293	5636 mg/L*	66.3mM*	See note below

*Note: this EC_{50} value exceeds the range of tested concentrations and may not be accurate. It was derived from the equation of the best-fit trendline and is presented for comparison purposes only.

Table 4.5: Calculated EC_{50} values for 24 hour ammonium nitrate resazurin assays in HepG2 and HEK293 cell lines. The EC_{50} values were calculated from the equation of the best-fit trendline of each experiment. The calculated EC_{50} values, expressed in mg/L of nitrate salt, were then converted to molar concentrations (mol/L). The EC_{50} is defined here as the nitrate concentration required to evoke 50% of the control fluorescence in the resazurin assay.

Exposure time & nitrate salt	**Cell type**	**Calculated EC_{50} (mg/L)**	**Calculated EC_{50} (mol/L)**	**Comments**
24h NH_4NO_3	HepG2	4789 mg/L	59.8mM	
	HEK293	3974 mg/L	49.6mM	

be significantly different from all others in this assay; some variation existed between the other flasks, but was not significant. Absorbance readings for the flasks ranged from a low of 0.183 to a high of 0.394; the latter reading was from the significantly different flask. The mean absorbance reading for all flasks was 0.273.

4.2.1.2 72 hour HepG2 media control group

The experimental set-up for this assay was identical to that described in section 4.2.1.1, except that the HepG2 cells were exposed to media for 72 hours. The results of this assay may be seen in Figure 4.16. No flasks were found to be significantly different from all others in this assay. There appeared to be less variability overall in the 72 hour assay compared to the 24 hour media control assay. Absorbance readings for the flasks ranged from a low of 1.226 to a high of 1.714, with an overall mean absorbance of 1.432.

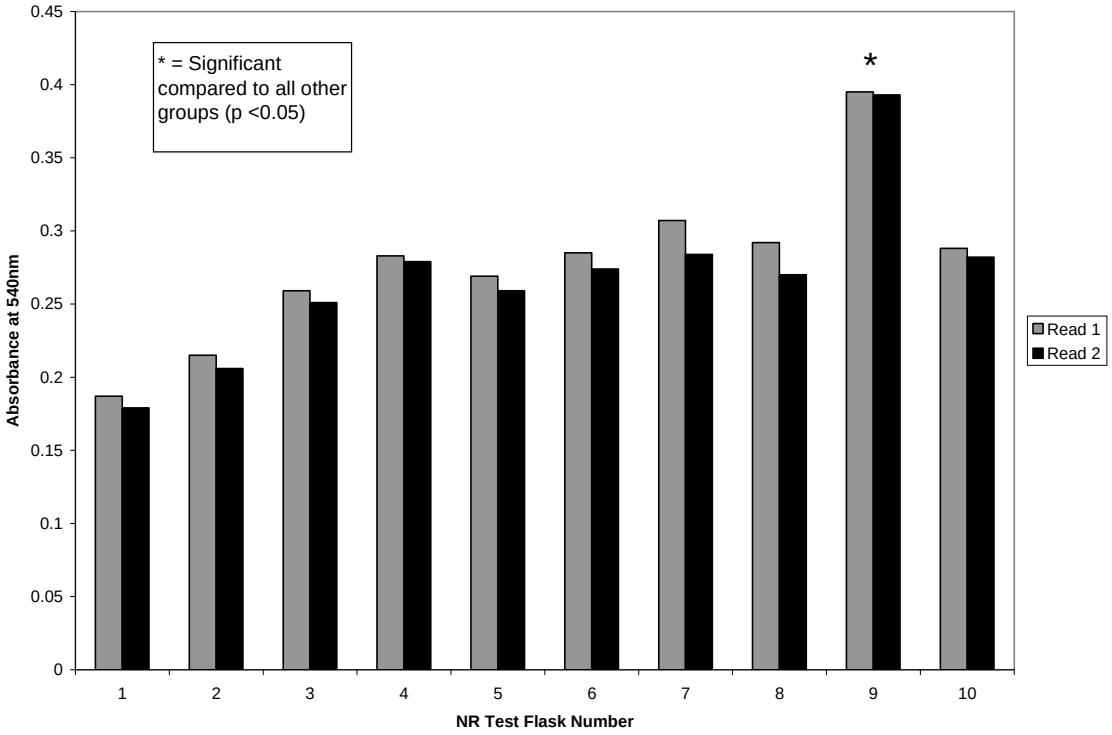


Figure 4.15: Absorbance at 540nm in HepG2 cells exposed to control media for 24 hours (Neutral Red assay). The HEK293 cell line was not tested in this assay. The absorbance of each sample was read twice; the first reading is expressed as a grey bar, while the second reading is represented by a black bar. The means of each set of readings were compared using one-way ANOVA, and each flask was compared to every other flask using the Bonferroni post-hoc analysis. A flask was deemed significantly different if it was compared to all other flasks and found to have a p-value below 0.05 for each. Significant differences to all other flasks were indicated with the * symbol. Only one flask (#9) was shown to be significantly different from all others in this assay. This flask was different from others at the following levels of significance: from #1 ($p=1.4x10^{-8}$), from #2 ($p=5.5x10^{-8}$), from #3 ($p=8.2x10^{-7}$), from #4 ($p=5.9x10^{-6}$), from #5 ($p=1.6x10^{-6}$), from #6 ($p=5.2x10^{-6}$), from #7 ($p=2.2x10^{-5}$), from #8 ($p=5.9x10^{-6}$), and from #10 ($p=8.4x10^{-6}$).

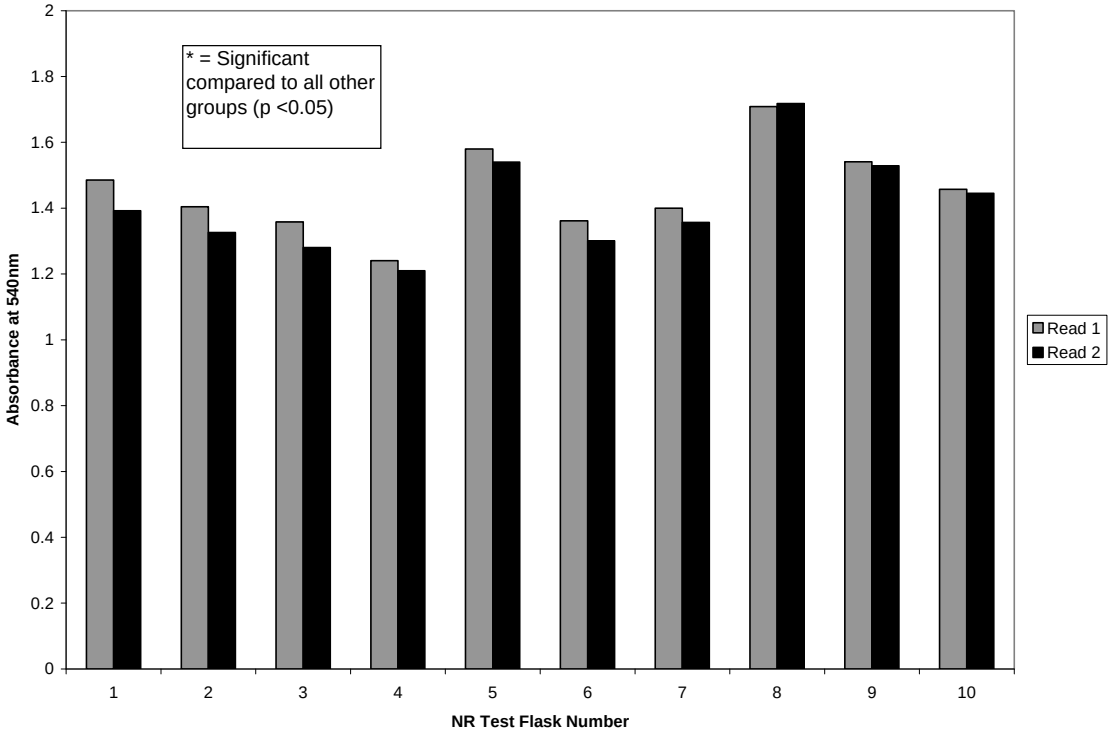


Figure 4.16: Absorbance at 540nm in HepG2 cells exposed to control media for 72 hours (Neutral Red assay). The HEK293 cell line was not tested in this assay. The absorbance of each sample was read twice; the first reading is expressed as a grey bar, while the second reading is represented by a black bar. The means of each set of readings were compared using one-way ANOVA, and each flask was compared to every other flask using the Bonferroni post-hoc analysis. A flask was deemed significantly different if it was compared to all other flasks and found to have a p-value below 0.05 for each. Significant differences to all other flasks were indicated with the * symbol. No flasks were found to be significantly different from all others in this assay.

4.2.1.3 48 hour HepG2 alternate salt exposure

To determine if the effects of potassium and ammonium nitrate exposure were due to the nitrate ion or the associated cation, a 48 hour Neutral Red assay with alternate salts was conducted on HepG2 cells. This assay utilized potassium acetate (CH_3COOK), ammonium acetate (CH_3COONH_4), and sodium nitrate ($NaNO_3$). Otherwise, the protocol was identical to that used for the experimental Neutral Red assays (see section 3.5). Figures were generated by pooling data from two replicate experiments. As seen in Figure 4.17, exposure to each alternate salt resulted in reduced viability with increasing salt concentration. An exponential decline in cell viability was observed in the potassium and ammonium acetate groups, while a cubic polynomial decline was observed in the sodium nitrate group. The overall sodium nitrate readings appeared to follow a sigmoidal curve. According to Figure 4.17, it appears that potassium acetate had the most deleterious effect on HepG2 cell viability, followed by ammonium acetate and sodium nitrate. Upon analysis with one-way ANOVA and Dunnett's post-hoc analysis, all alternate salts were found to decrease cell viability significantly ($p<0.05$) compared to the respective control groups at concentrations at and above 3500 mg/L. Additional significant differences from control viability were observed in sodium nitrate at 3000 mg/L $NaNO_3$, and in potassium acetate at 1500 mg/L, 2000 mg/L, 2500 mg/L, and 3000 mg/L CH_3COOK. The approximate EC_{50} values for 48 hour alternate salt exposure in HepG2 cells were 1773 mg/L (CH_3COOK), 3577 mg/L (CH_3COONH_4), and 5820 mg/L ($NaNO_3$) (see Table 4.3).

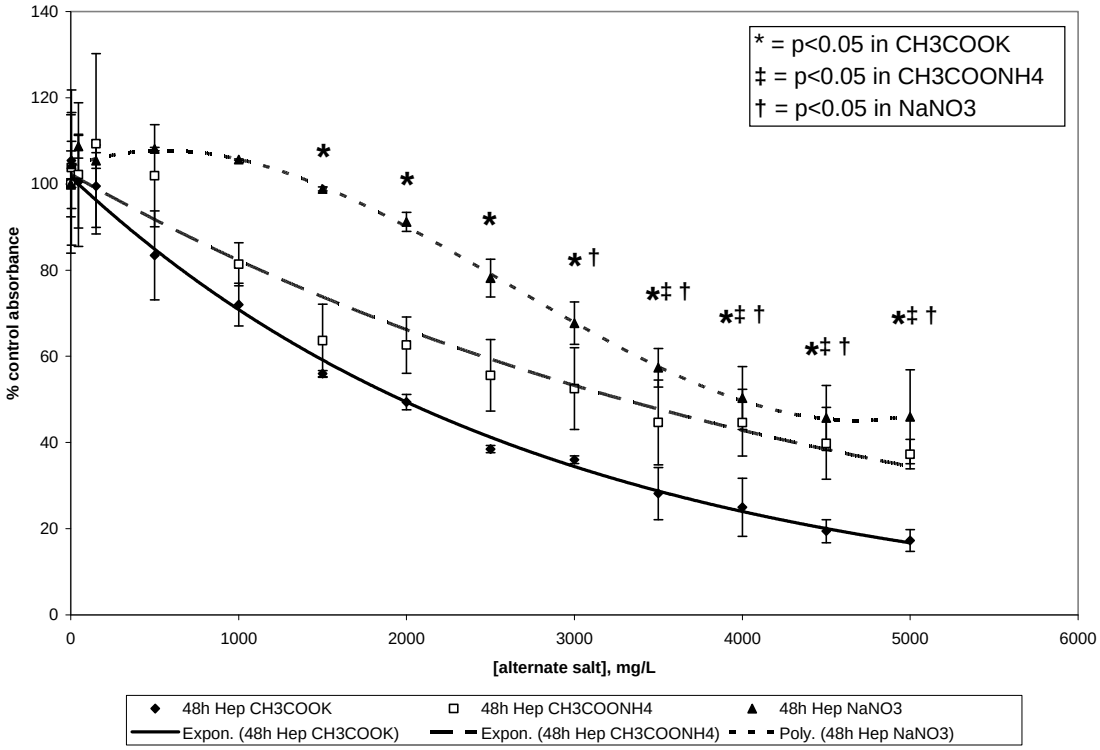


Figure 4.17: Percentage of control absorbance at 540nm in HepG2 cells as a function of potassium acetate, ammonium acetate, or sodium nitrate concentration in a 48 hour exposure (Neutral Red assay). The HEK293 cell line was not tested in this assay. Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). A closed diamond (◆) with a solid trendline was used to signify 48h CH_3COOK data, an open square (□) with a dashed trendline was used to signify 48h CH_3COONH_4 data, and a closed triangle (▲) with a dotted trendline was used to signify 48h $NaNO_3$ data. Significant ($p<0.05$) differences from control absorbance are denoted by the * symbol for the 48h CH_3COOK exposure, the ‡ symbol for the 48h CH_3COONH_4 exposure, and the † symbol for the 48h $NaNO_3$ exposure. Significant differences from control absorbance were observed in the HepG2 cells exposed to CH_3COOK for 48 hours at the following concentrations: 1500 mg/L (p=0.003), 2000 mg/L (p=0.0009), 2500 mg/L (p=0.0001), 3000 mg/L (p=0.0001), 3500 mg/L (p=0.00002), 4000 mg/L (p=0.00001), 4500 mg/L (p=0.00001), and 5000 mg/L (p=0.000005). Significant differences from control absorbance were observed in HepG2 cells exposed to CH_3COONH_4 for 48 hours at the following concentrations: 3500 mg/L (p=0.042), 4000 mg/L (p=0.042), 4500 mg/L (p=0.025), and 5000 mg/L (p=0.019). Significant differences from control absorbance were observed in HepG2 cells exposed to $NaNO_3$ for 48 hours at the following concentrations: 3000 mg/L (p=0.003), 3500 mg/L (p=0.0003), 4000 mg/L (p=0.0001), 4500 mg/L (p=0.00002), and 5000 mg/L (p=0.00002).

4.2.1.4 48 hour HEK293 alternate salt exposure

The experimental set-up for this assay was identical to that for the 48 hour HepG2 alternate salt exposure (see section 4.2.1.3), except that potassium acetate was not used for HEK293 exposures. A highly divergent response was observed between the HEK293 cells exposed to ammonium acetate and sodium nitrate, as seen in Figure 4.18. While the HEK293 cells experienced a strong cubic polynomial decline in cell viability with increasing CH_3COONH_4 concentration, only a slight linear decline in viability was observed in cells exposed to $NaNO_3$. Analysis with one-way analysis of variance (one-way ANOVA) and Dunnett's post-hoc analysis revealed significant differences compared to control group viability in the CH_3COONH_4 group at concentrations at and above 500 mg/L. No significant differences compared to control group viability were observed in HEK293 cells exposed to $NaNO_3$ in a 48 hour exposure. The approximate EC_{50} value for a 48 hour alternate salt exposure in HEK293 cells was 1328 mg/L for CH_3COONH_4. The $NaNO_3$ EC_{50} was not calculable from the available data due to the minor effect of sodium nitrate on HEK293 cell viability (see Table 4.3).

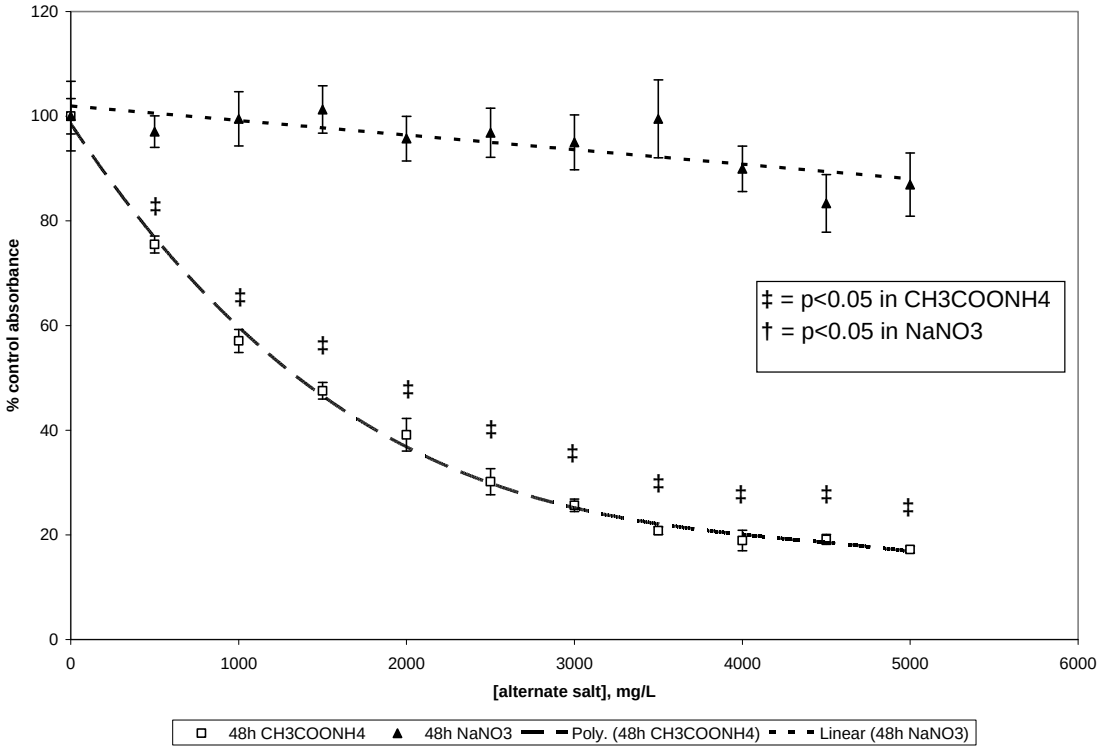


Figure 4.18: Percentage of control absorbance at 540nm in HEK293 cells as a function of ammonium acetate or sodium nitrate concentration in a 48 hour exposure (Neutral Red assay). The HepG2 cell line was not tested in this assay. Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). An open square (□) with a dashed trendline was used to signify 48h CH_3COONH_4 data, and a closed triangle (▲) with a dotted trendline was used to signify 48h $NaNO_3$ data. Significant ($p<0.05$) differences from control absorbance are denoted by the ‡ symbol for the 48h CH_3COONH_4 exposure and the † symbol for the 48h $NaNO_3$ exposure. Significant differences from control absorbance were observed in HEK293 cells exposed to CH_3COONH_4 for 48 hours at the following concentrations: 500 mg/L ($p=1.5x10^{-7}$), 1000 mg/L ($p=2.1x10^{-9}$), 1500 mg/L ($p=2.1x10^{-9}$), 2000 mg/L ($p=2.1x10^{-9}$), 2500 mg/L ($p=2.1x10^{-9}$), 3000 mg/L ($p=2.1x10^{-9}$), 3500 mg/L ($p=2.1x10^{-9}$), 4000 mg/L ($p=2.1x10^{-9}$), 4500 mg/L ($p=2.1x10^{-9}$), and 5000 mg/L ($p=2.1x10^{-9}$). No significant differences from control absorbance were observed in the HEK293 cells exposed to sodium nitrate for 48 hours.

4.2.2 5-bromo-2'-deoxyuridine Enzyme-Linked Immunosorbent Assay (BrdU ELISA) alternate salt assay results

4.2.2.1 48 hour HepG2 alternate salt exposure

As with the Neutral Red assays described in section 4.2.1.3, BrdU ELISA assays were run to determine the effect of different potassium, ammonium, and nitrate salts on HepG2 cell proliferation. Potassium acetate (CH_3COOK), ammonium acetate (CH_3COONH_4), and sodium nitrate ($NaNO_3$) were used in this assay. Other than the salt substitutions, the experimental protocol was identical to the BrdU ELISA protocol described in section 3.6. Because of high inter-experiment variability, representative traces – not pooled data – were used to generate figures. Representative traces were chosen as described in section 4.1.2. As seen in Figure 4.19, potassium and ammonium acetate appeared to have near-identical effects on cell proliferation, while sodium nitrate appeared to have little deleterious effect. The potassium and ammonium acetate-treated groups displayed strong exponential decreases in cell proliferation with increasing salt concentration. However, sodium nitrate showed a weak exponential decline with increasing $NaNO_3$ concentration; proliferation remained at 60% of control group levels even at 5000 mg/L $NaNO_3$. All treatment groups showed an increase in cell proliferation at low salt concentrations; most readings up to 250 mg/L in each salt showed proliferation greater than 100% of control group values. Analysis with one-way analysis of variance (one-way ANOVA) and Dunnett's post-hoc analysis revealed viability significantly different ($p<0.05$) than the control group at several nitrate exposure concentrations. As a result of the high proliferation observed at low salt concentrations, some experimental groups show significantly *increased* proliferation than their respective control groups. These included 45 mg/L for CH_3COOK; 1 µg/L, 3 mg/L, and

250 mg/L for CH_3COONH_4; and 45 mg/L and 250 mg/L for $NaNO_3$. Significantly *decreased* proliferation compared to control group was observed in HepG2 cells exposed to concentrations greater than or equal to 1500 mg/L in HepG2 cells treated with CH_3COOK and concentrations greater than or equal to 500 mg/L in HepG2 cells treated with CH_3COONH_4. Significantly decreased proliferation compared to the control group was not observed in HepG2 cells exposed to sodium nitrate at concentrations used in this experiment, and the EC_{50} value for this assay could not be accurately determined from the available data. The approximate EC_{50} values for HepG2 cells exposed to the other alternate salts for 48 hours were 1248 mg/L for CH_3COOK, and 943 mg/L for CH_3COONH_4 (see Table 4.4).

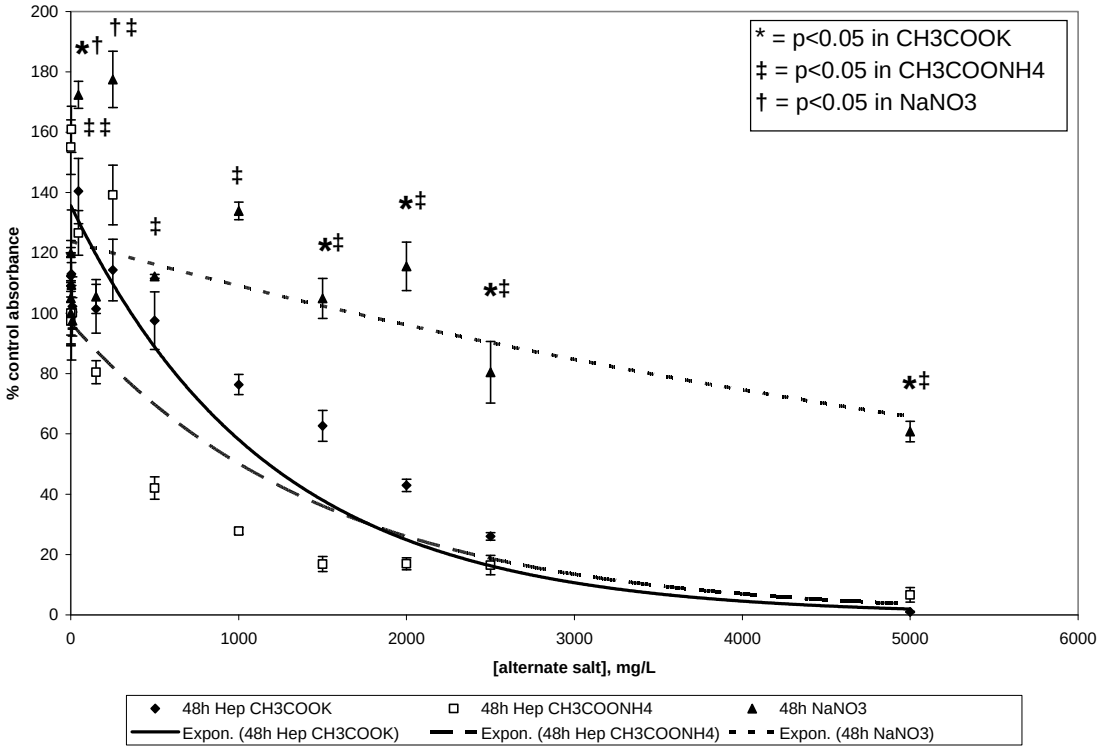


Figure 4.19: Percentage of control absorbance at 450nm in HepG2 cells as a function of potassium acetate, ammonium acetate, or sodium nitrate concentration in a 48 hour exposure (BrdU ELISA). The HEK293 cell line was not tested in this assay. This graph contains representative traces from each experiment, not pooled data. Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). A closed diamond (◆) with a solid trendline was used to signify 48h CH_3COOK data, an open square (□) with a dashed trendline was used to signify 48h CH_3COONH_4 data, and a closed triangle (▲) with a dotted trendline was used to signify 48h $NaNO_3$ data. Significant ($p<0.05$) differences from control absorbance are denoted by the * symbol for the 48h CH_3COOK exposure, the ‡ symbol for the 48h CH_3COONH_4 exposure, and the † symbol for the 48h $NaNO_3$ exposure. Significant differences from control absorbance were observed in the HepG2 cells exposed to CH_3COOK for 48 hours at the following concentrations: 45 mg/L ($p=0.01$), 1500 mg/L ($p=0.023$), 2000 mg/L ($p=8.8\times10^{-5}$), 2500 mg/L ($p=3.0\times10^{-7}$), and 5000 mg/L (2.1×10^{-8}). Significant differences from control absorbance were observed in HepG2 cells exposed to CH_3COONH_4 for 48 hours at the following concentrations: 1µg/L ($p=1.3\times10^{-6}$), 3 mg/L ($p=1.1\times10^{-7}$), 250 mg/L ($p=0.001$), 500 mg/L ($p=3.6\times10^{-7}$), 1000 mg/L ($p=2.2\times10^{-8}$), 1500 mg/L ($p=2.1\times10^{-8}$), 2000 mg/L ($p=2.1\times10^{-8}$), 2500 mg/L ($p=2.1\times10^{-8}$), and 5000 mg/L ($p=2.1\times10^{-8}$). Significant differences from control absorbance were observed in HepG2 cells exposed to $NaNO_3$ for 48 hours at the following concentrations: 45 mg/L ($p=0.007$), and 250 mg/L ($p=0.003$).

4.2.2.2 48 hour HEK293 alternate salt exposure

The experimental set-up for this assay was identical to that for the 48 hour HepG2 alternate salt exposure (see section 4.2.2.1), except that potassium acetate was not used for HEK293 exposures. As seen in Figure 4.20, the responses of the HEK293 cells to ammonium acetate and sodium nitrate were dissimilar. Although both salts induced exponentially-decreasing cell proliferation with increasing salt concentration, the CH_3COONH_4 group appeared to decline more rapidly than the $NaNO_3$ group. Exposure to low concentrations of CH_3COONH_4 also appeared to evoke an increased degree of cell proliferation compared to the control group; however, this increase was not statistically significant. Analysis with one-way analysis of variance (one-way ANOVA) and Dunnett's post-hoc analysis revealed that both salts induced significantly ($p<0.05$) decreased cell proliferation compared to the control groups at concentrations at and above 1500 mg/L. Additional significantly decreased cell proliferation was observed in HEK293 cells exposed to ammonium acetate at 500 mg/L and 1000 mg/L CH_3COONH_4. At ammonium nitrate concentrations at and above 2500 mg/L, cell proliferation was less than 10% of control. Conversely, cell proliferation was 45% of control in HEK293 cells exposed to 5000 mg/L of sodium nitrate. Approximate EC_{50} values for HEK293 cells exposed to alternate salts for 48 hours were 907 mg/L for CH_3COONH_4 and 5636 mg/L for $NaNO_3$ (see Table 4.4).

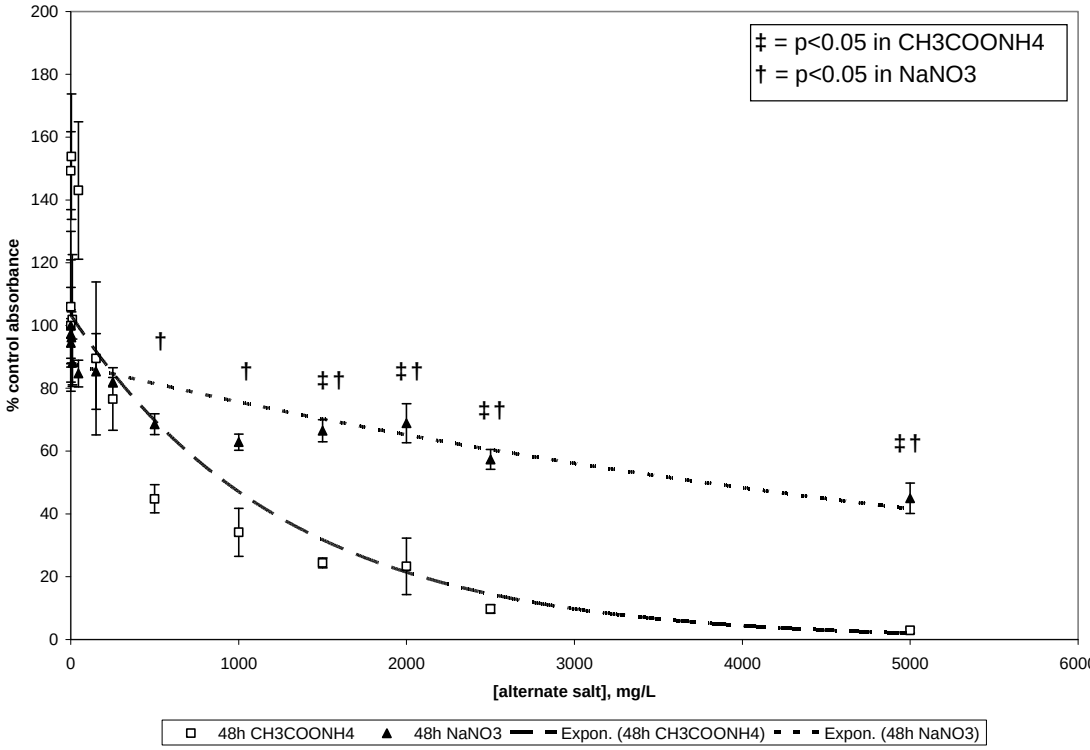


Figure 4.20: Percentage of control absorbance at 450nm in HEK293 cells as a function of ammonium acetate or sodium nitrate concentration in a 48 hour exposure (BrdU ELISA). The HepG2 cell line was not tested in this assay. This graph contains representative traces from each experiment, not pooled data. Absorbances were expressed as a percentage of control group absorbance, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). An open square (□) with a dashed trendline was used to signify 48h CH_3COONH_4 data, and a closed triangle (▲) with a dotted trendline was used to signify 48h $NaNO_3$ data. Significant ($p<0.05$) differences from control absorbance are denoted by the ‡ symbol for the 48h CH_3COONH_4 exposure and the † symbol for the 48h $NaNO_3$ exposure. Significant differences from control absorbance were observed in HEK293 cells exposed to CH_3COONH_4 for 48 hours at the following concentrations: 1500 mg/L ($p=0.021$), 2000 mg/L ($p=0.018$), 2500 mg/L ($p=0.003$), and 5000 mg/L ($p=0.001$). Significant differences from control absorbance were observed in HEK293 cells exposed to $NaNO_3$ for 48 hours at the following concentrations: 500 mg/L ($p=0.029$), 1000 mg/L ($p=0.006$), 1500 mg/L ($p=0.016$), 2000 mg/L ($p=0.031$), 2500 mg/L ($p=0.001$), and 5000 mg/L ($p=9.9\times10^{-5}$).

4.2.3 24 hour ammonium nitrate resazurin viability assay

To verify the results of the Neutral Red assays, HepG2 and HEK293 cells exposed to ammonium nitrate for 24 hours were analyzed using the resazurin assay protocol, as described in section 3.7. The resazurin assay provides a metabolism-based measure of cell viability, as opposed to the Neutral Red dye-uptake mechanism (see sections 3.5 and 3.7). The results of this assay may be seen in Figure 4.21. Only one experiment, consisting of HepG2 and HEK293 cells in a single cell culture plate, was performed and used to generate this figure. Both the HepG2 and HEK293 cells showed a decrease in cell viability with increasing ammonium nitrate concentration. A large decrease in cell viability was visible in both cell types at and above 4500 mg/L NH_4NO_3. Both data sets were found to follow a quadratic polynomial trendline. After analysis with one-way ANOVA and Dunnett's post-hoc analysis, viability was found to be significantly ($p<0.05$) decreased compared to control at concentrations greater than and equal to 3500 mg/L NH_4NO_3 in both cell types. Viability was also significantly decreased in HEK293 cells at 1000 mg/L and 1500 mg/L NH_4NO_3, and in HepG2 cells at 3 mg/L and 500 mg/L NH_4NO_3. The approximate EC_{50} values for 24 hour ammonium nitrate exposure in this experiment were 4789 mg/L in HepG2 cells and 3974 mg/L in HEK293 cells, as seen in Table 4.5.

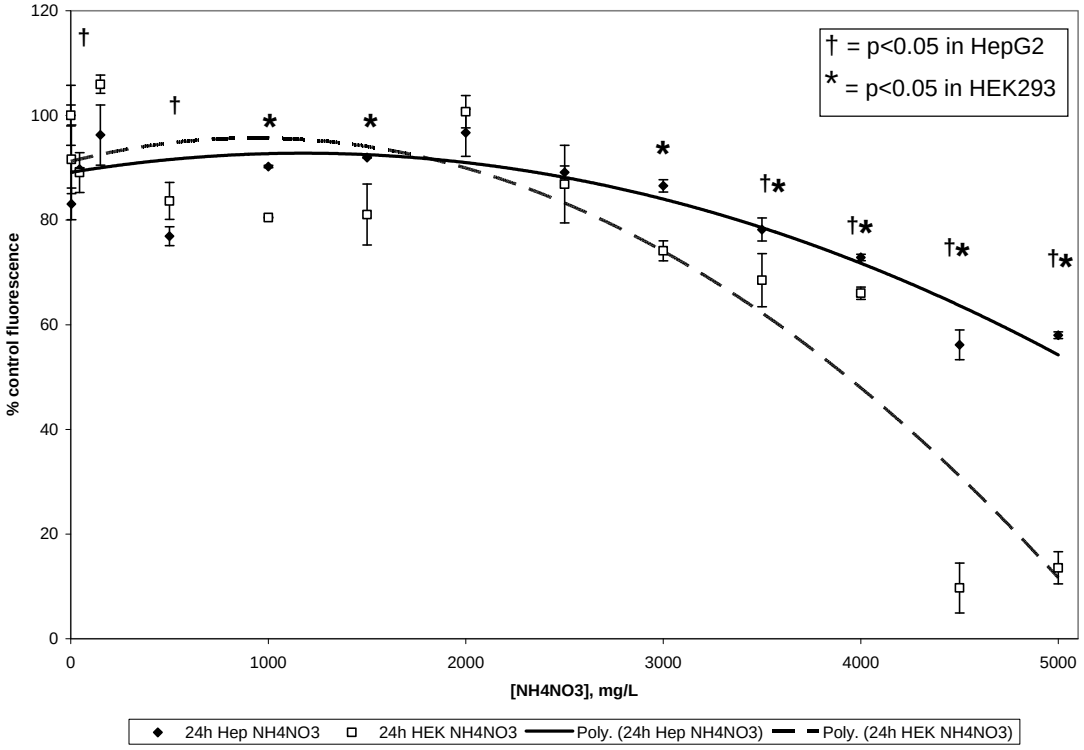


Figure 4.21: Percent of control fluorescence at 590nm in HepG2 and HEK293 cells as a function of ammonium nitrate concentration in a 24 hour exposure (Resazurin assay). This graph contains pooled data from one replicate plate. Fluorescences were expressed as a percentage of control group fluorescence, then means were compared using one-way ANOVA and two-sided Dunnett's post-hoc analysis. Data are expressed as +/- standard error of the mean (SEM). A closed diamond (◆) with a solid trendline was used to signify HepG2 data, while an open square (□) with a dashed trendline was used to signify HEK293 data. Significant ($p<0.05$) differences from control fluorescence are denoted by the † symbol for HepG2 cells and the * symbol for HEK293 cells. Significant differences from control fluorescence were observed in HepG2 cells at the following concentrations: 3 mg/L ($p=0.018$), 500 mg/L ($p=0.001$), 3500 mg/L ($p=0.001$), 4000 mg/L ($p=7.1\times10^{-5}$), 4500 mg/L ($p=2.4\times10^{-8}$), and 5000 mg/L ($p=3.8\times10^{-8}$). Significant differences from control fluorescence were observed in HEK293 cells at the following concentrations: 1000 mg/L ($p=0.02$), 1500 mg/L ($p=0.029$), 3000 mg/L ($p=0.001$), 3500 mg/L ($p=9.7\times10^{-5}$), 4000 mg/L ($p=3.0\times10^{-5}$), 4500 mg/L ($p=1.5\times10^{-8}$), and 5000 mg/L ($p=1.5\times10^{-8}$).

4.3 Nitrate and protein expression: Western blotting results

All Western blots were generated as described in section 3.10. The resulting films were marked with permanent ink to indicate the position of the molecular weight ladder and the antibody applied. Films were then digitized via scanning, and cropped to show only the band of interest. All scanned copies were compared to the original films to ensure correct orientation of the bands.

4.3.1 Proliferating Cell Nuclear Antigen (PCNA) results

A slight change in protein expression over the range of tested nitrate concentrations was noted for both HepG2 and HEK293 cells. In HepG2 cells, a slight increase in Western blot band intensity was noted for both potassium and ammonium nitrate exposures (Figure 4.22). This effect is noticeable in both salts tested but appears to be more intense in the ammonium nitrate blot. For HEK293 cells, a slight decrease in band intensity was visible with increasing nitrate concentrations. This decrease was seen in both potassium and ammonium nitrate exposures. All bands visualized at an approximate molecular weight of 36kDa, equal to the weight of the PCNA protein.

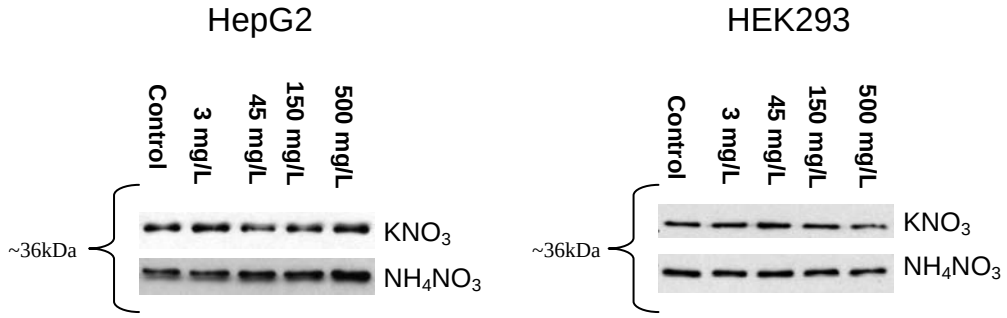


Figure 4.22: Proliferating Cell Nuclear Antigen (PCNA) Western blot films of HepG2 and HEK293 cells exposed to potassium and ammonium nitrate. Cells of each type were treated either with 0 mg/L (control), 3 mg/L, 45 mg/L, 150 mg/L, or 500 mg/L of potassium or ammonium nitrate. Resulting bands were observed at approximately 36kDa, the molecular weight of the PCNA protein. Band intensity appeared to increase with increasing nitrate concentration in HepG2 cells (blots at left), but decreased with increasing nitrate concentration in HEK293 cells (blots at right). Each set of bands was a separate Western blot experiment. Each experiment was performed twice to ensure validity of findings.

4.3.2 Heat Shock Protein 70 (Hsp70) results

All cell types and nitrate salts tested showed a very slight elevation in Hsp70 protein expression with increasing nitrate concentration (Figure 4.23). Both HepG2 and HEK293 cells experienced the increase, but due to greater band resolution the change was more apparent in the HEK293 Western blots. In HepG2 cells, the increase is more evident in the potassium nitrate group than the ammonium nitrate group, which may be due to better band resolution. In HEK293 cells, both potassium and ammonium nitrate groups appear to show a change of similar magnitude. All bands visualized at an approximate molecular weight of 70kDa, which is equal to the weight of the Hsp70 protein.

4.3.3 Heat Shock Cognate Protein 70 (Hsc70) results

None of the cell types or nitrate salts tested showed a visible change in Hsc70 protein levels at any of the nitrate concentrations (Figure 4.24). Changes were not evident between nitrate salts (KNO_3 and NH_4NO_3) or cell types. All bands visualized at an approximate molecular weight of 70kDa, which is approximately equal to the weight of the Hsc70 protein.

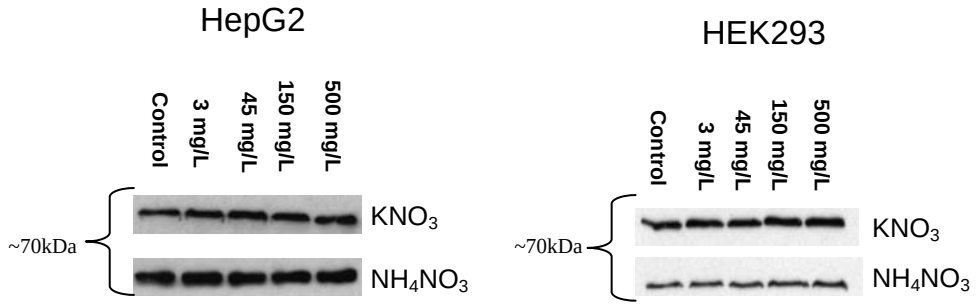


Figure 4.23: Heat Shock Protein 70 (Hsp70) Western blot films of HepG2 and HEK293 cells exposed to potassium and ammonium nitrate. Cells of each type were treated either with 0 mg/L (control), 3 mg/L, 45 mg/L, 150 mg/L, or 500 mg/L of potassium or ammonium nitrate. Resulting bands were observed at approximately 70kDa, the molecular weight of the Hsp70 protein. Band intensity appeared to increase slightly in both HepG2 cells (blots at left), and HEK293 cells (blots at right) with increasing nitrate concentrations. Each set of bands was a separate Western blot experiment. Each experiment was performed twice to ensure validity of findings.

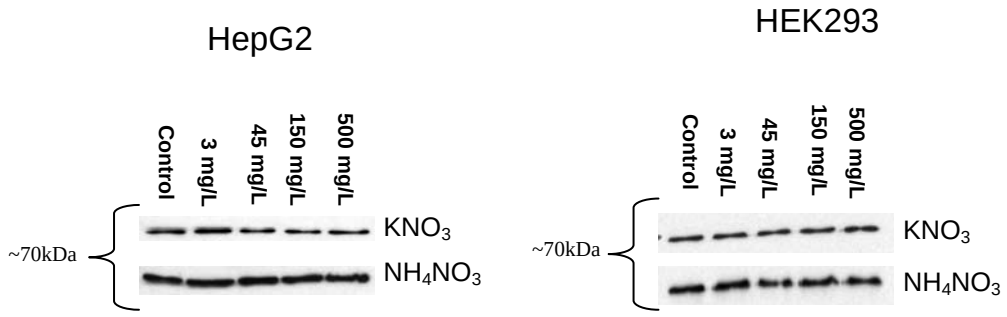


Figure 4.24: Heat Shock Cognate Protein 70 (Hsc70) Western blot films of HepG2 and HEK293 cells exposed to potassium and ammonium nitrate. Cells of each type were treated either with 0 mg/L (control), 3 mg/L, 45 mg/L, 150 mg/L, or 500 mg/L of potassium or ammonium nitrate. Resulting bands were observed at approximately 70kDa, the approximate molecular weight of the Hsc70 protein. Band intensity did not appear to change in either HepG2 cells (blots at left) or HEK293 cells (blots at right) with increasing nitrate concentrations. Each set of bands was a separate Western blot experiment. Each experiment was performed twice to ensure validity of findings.

4.3.4 Vascular Endothelial Growth Factor (VEGF) results

Due to difficulties visualizing the VEGF protein in HepG2 cells, only protein expression data for HEK293 cells is available. In addition, only one blot from each nitrate salt was obtainable. There did not appear to be any change in protein expression with increasing nitrate concentration (Figure 4.25); however, the blots were very faint so determining relative change was difficult. The 45 mg/L band on the potassium nitrate blot appears to be more intense than the surrounding bands, but this is likely due to loading or exposure issues rather than an actual change in protein expression. The ammonium nitrate blot was visible on the original film, but the intensity was insufficient to display after scanning; however, no change in intensity was apparent. All bands visualized at an approximate molecular weight of 21kDa, which is approximately equal to the weight of the VEGF protein.

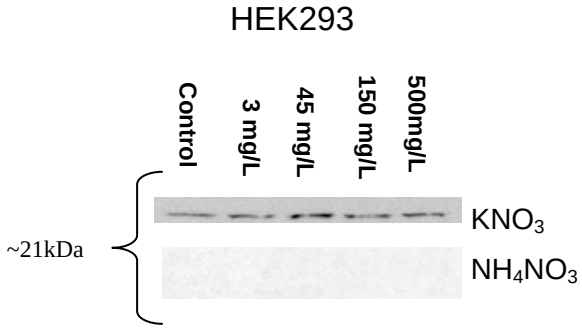


Figure 4.25: Vascular Endothelial Growth Factor (VEGF) Western blot films of HEK293 cells exposed to potassium and ammonium nitrate. Due to difficulties in visualizing the protein, data for HepG2 cells were not obtainable. Cells were treated either with 0 mg/L (control), 3 mg/L, 45 mg/L, 150 mg/L, or 500 mg/L of potassium or ammonium nitrate. Resulting bands were observed at approximately 21kDa, the approximate molecular weight of the VEGF protein. Band intensity did not appear to change in the HEK293 cells with increasing nitrate concentrations. Bands were present in the NH_4NO_3 blot; however, the intensity was insufficient to visualize upon scanning. The intensity of the NH_4NO_3 bands did not appear to change with increasing nitrate concentration. However, it should be noted that the bands were very faint and visualization was difficult. Each set of bands was a separate Western blot experiment. The VEGF blot was performed only once for each salt due to difficulties in detecting and visualizing the protein.

CHAPTER 5: DISCUSSION

5.1 Cytotoxic effects of nitrate

5.1.1 Effects of nitrate on cell viability

In these experiments, a variety of responses to nitrate exposure were noted in HepG2 and HEK293 cells. These responses varied with cell type, nitrate salt, nitrate concentration, and exposure time. Each individual parameter, as well as possible explanations for the observed results, are described below.

5.1.1.1 Effects of nitrate on HepG2 vs. HEK293 viability

Based on the results of the Neutral Red viability assays, it appears that HepG2 and HEK293 cells did react differently to nitrate exposure in some assays. In both ammonium nitrate assays (24 and 72 hours), the HepG2 and HEK293 cells behaved in a similar manner; both experienced declines of similar magnitude as the NH_4NO_3 concentration increased. The HEK293 cells did appear to be slightly more sensitive to NH_4NO_3 during the 24 hour NH_4NO_3 assay; although the shape of the trendlines was similar, the HEK293 viability was consistently lower than the HepG2 viability at most of the NH_4NO_3 concentrations tested. This finding was not observed in the 72 hour NH_4NO_3 assay. Thus, it is possible to conclude that HEK293 cells may experience a slightly greater decline in viability when exposed to NH_4NO_3 in short-term exposures compared to HepG2 cells under these experimental conditions. In longer-term

exposures, however, there does not appear to be a substantial difference in the effect of NH_4NO_3 on viability in HepG2 and HEK293 cells.

However, a definite difference in response was observed between the HepG2 and HEK293 cell types in both KNO_3 assays. While HepG2 cells experienced a decline in viability in both assays, the HEK293 cell viability increased in the 24 hour KNO_3 exposure and only slightly decreased in the 72 hour KNO_3 exposure. The reasons for this discrepancy are unclear, particularly for the increase in viability observed in the 24 hour KNO_3 exposure.

One potential explanation for the unique HEK293 response is that the observed increase in viability is actually an artifact. It is possible that a property unique to HEK293 cells resulted in greater accumulation of Neutral Red dye, causing them to appear more viable. This may have resulted from changes in the cell membrane triggered by the high experimental nitrate concentrations, or some other characteristic unique to HEK293 cells. In the short term, this may have increased the uptake of Neutral Red dye; in the long term, however, the membrane alteration may have gradually resulted in cell death. This hypothesis could explain why an increase was seen in the 24 hour KNO_3 exposure, but a slight decrease was seen in the 72 hour KNO_3 exposure (see section 5.1.1.3 for further discussion). Some evidence indicates that high potassium exposure can change membrane properties; Ohba *et al*. (1994) found that exposure to a 10 mM potassium solution decreased rat neuronal membrane fluidity.

Although this is a possible explanation for these observations, other factors do not support this hypothesis. While exposure to high concentrations of potassium has been shown to decrease membrane fluidity (Ohba *et al*., 1994), experiments with artificial cell membranes have shown that decreases in membrane fluidity usually results in reduced permeability (Lande *et al*., 1995). Thus, a *decrease* in permeability to Neutral Red dye would be expected if potassium-mediated changes to the cell membrane were occurring. However, because this was not seen in the KNO_3 Neutral Red experiments, another process must be responsible for the increased readings. The mechanism of Neutral Red uptake also provides contradictory evidence to the hypothesis of greater HEK293 membrane permeability alone, as described below.

Neutral Red dye is an acidotropic weak base which is not membrane-permeable in its cationic state. Under physiological conditions, such as in cell culture, an equilibrium forms between the charged and uncharged forms of the dye. Approximately half of the Neutral Red will then be in the membrane-permeable uncharged free base form. The uncharged Neutral Red freely diffuses through the cell and lysosomal membranes, but when it encounters the acidic lysosomal environment, it reverts back into the non-permeable cationic form and is trapped inside the lysosome (Rashid *et al*., 1991). Because the dye is a weak base, placing the dye into a slightly basic environment should shift the equilibrium towards the uncharged free base form. Solutions of KNO_3 are essentially neutral, even at high concentrations. However, solutions of NH_4NO_3 do become more basic with increasing NH_4NO_3 concentration. Since NH_4NO_3 produces a more basic solution than KNO_3, it would be expected that more Neutral Red would be in the uncharged form in a NH_4NO_3 solution compared to a KNO_3 solution. This would in

turn result in greater Neutral Red penetration of the cell and lysosomal membranes, and greater retention of the dye in the cells. Thus, if HEK293 cells were more permeable to Neutral Red than HepG2 cells, and this was the only parameter considered, it would be expected to see greater dye retention in the ammonium nitrate group compared to the potassium nitrate group in the HEK293 exposures. Because of the more favourable conditions for Neutral Red dye penetration in ammonium nitrate exposures, it might also be anticipated that HepG2 NH_4NO_3 exposures would show greater dye retention, though not as much as HEK293 cells. This was not seen in these experiments, as the 24 hour NH_4NO_3 exposure indicated that HEK293 cells were slightly *more* sensitive to nitrate toxicity (i.e. retained less Neutral Red dye) than the HepG2 cells. Due to the results of these experiments, it does not appear that cell type alone explains the discrepancy observed between HepG2 and HEK293 cells. However, cell type may have played a role in conjunction with other experimental variables, such as the choice of nitrate salt.

5.1.1.2 Effects of potassium nitrate vs. ammonium nitrate on cell viability

The Neutral Red assays have also demonstrated that ammonium and potassium nitrate evoke different patterns of toxicity in HepG2 and HEK293 cell viability. Generally, the ammonium nitrate assays showed a greater reduction in viability for both HepG2 and HEK293 cells than the potassium nitrate assays. Examination of Table 4.1 shows that the EC_{50} values for each NH_4NO_3 exposure are lower than the KNO_3 exposure at the same timepoint for both HepG2 and HEK293 cells. The trendlines of the ammonium nitrate exposure graphs (see Figures 4.1 through 4.10) appear to be more depressed and show a more rapid decline in cell viability than the potassium nitrate exposure trendlines. Thus, it is possible to conclude that ammonium nitrate had a greater

detrimental effect on both HepG2 and HEK293 cell viability in these experiments than potassium nitrate. As discussed previously, the HepG2 and HEK293 cells responded similarly to NH_4NO_3 exposure, with the HEK293 cells appearing slightly more sensitive during the 24 hour exposures but not the 72 hour exposures.

Several factors may explain the greater toxicity of ammonium nitrate in these assays. As discussed in section 5.1.1.1, a small difference in pH exists between the potassium and ammonium nitrate solutions. While it is unlikely that a change in pH evoked by the addition of an aliquot of ammonium nitrate into a cell culture would be fatal to cells, due to the effects of dilution and the presence of sodium bicarbonate buffer, it may represent an additional stress and further increase cell susceptibility to the cytotoxic effects of nitrate. From the differences in toxicity observed in the potassium and ammonium salt groups, it is likely that the cation coupled with the nitrate anion contributes to the toxicity observed in the Neutral Red assay. This is supported by the observation that only the potassium salt evoked large differences in viability between the two cell types; the ammonium salt produced relatively similar responses in both the HepG2 and HEK293 cells. The presence and concentration of potassium ions in the KNO_3 exposure may have modified the toxicity of nitrate to the HEK293 cells, while the presence and concentration of ammonium ions in the NH_4NO_3 exposure may have increased toxicity to both cell types. Thus, it appears that an interaction between cell type and the cation paired with nitrate may explain the toxicity observed in these studies.

There are several mechanisms by which a potassium salt may have affected the HEK293 cells differently than an ammonium nitrate salt. One potential mechanism is the

alteration of cell membrane potential from the high concentrations of potassium ions in the KNO_3 exposure. Normally, the intracellular potassium concentration of mammalian cells (140 mM) is much greater than the extracellular potassium concentration (5 mM) (Cooper, 2000); however, the KNO_3 solutions used in these experiments would have greatly increased the extracellular potassium concentration. In turn, this could cause a change in the membrane potential, affecting structures such as voltage-gated ion channels or the Na^+/K^+/ATPase ion pump. This could then result in imbalances of intracellular ion concentrations and cellular dysfunction. Although Neutral Red uptake occurs by passive diffusion, and thus would likely not be significantly affected by the activity of voltage-gated ion channels, a change in membrane potential and cell homeostasis might result in conditions more favourable for the retention of Neutral Red dye. The specific details of such changes and their resulting conditions is beyond the scope of this thesis, but the potential for these situations should be considered. However, a disruption in membrane potential caused by hyperkalemia would logically affect both cell types, not just the HEK293 cells. Thus, although a disruption in membrane potential may partially explain the results observed in these assays, some feature unique to the HEK293 cells must also be responsible.

Another potential mechanism could involve the physiological interaction between renal cells and potassium concentrations. Kidney cells, such as the HEK293 cell line, have unique and specialized functions. One important renal function is the regulation of potassium within the body. In human adults, the balance of potassium in the body is controlled almost exclusively by the kidney (Valtin & Schafer, 1995). As stated previously, a large difference exists between extracellular (5 mM) and intracellular (140

mM) potassium concentrations. The maintenance of low potassium concentration in the extracellular fluid (ECF) is very important for proper function of excitable tissues, such as nerve and muscle cells. Even a slight increase in ECF potassium concentration, from approximately 5 mM to 6.5 mM, is considered "dangerous" (Valtin & Schafer, 1995).

Despite the need for tight control of potassium concentrations within the body, potassium intake *in vivo* varies widely with diet. Kidney cells are adapted to withstand large fluctuations in potassium concentration, as evidenced by the ability of most healthy adults to withstand a 10-fold increase in potassium intake with no adverse effects (Lote, 2000). A blood transfusion may introduce as much as 30 mM of potassium into the body, yet adverse effects in adults are rare (Lote, 2000). Upon introduction of excess potassium into the body, it is filtered into the nephron and secreted into the urine. *In vivo*, potassium may be temporarily partitioned into muscle and liver tissue in order to lower serum potassium concentrations (Malnic *et al*., 2004); however, this mechanism would not be possible in cell culture. Excess potassium induces an increase in amount and activity of sodium-potassium ATPase (Na^+/K^+/ATPase), which exchanges three sodium ions out of the kidney cell for two potassium ions into the cell. High potassium concentrations also result in an increase of potassium channels and an increase in membrane permeability to potassium in renal cells (Lote, 2000). The end result of these modifications is to increase the flow of potassium from the blood into the urine in order to maintain homeostasis in the body.

It is possible that this upregulation of potassium handling mechanisms is activated during exposures of kidney cell cultures to high concentrations of potassium, as in this

study. This upregulation may have conferred increased resistance to the toxic effects of nitrate, though the mechanism by which this might occur is unclear. Alternately, the increase in membrane permeability to potassium may have also increased Neutral Red permeability, allowing more of the dye to enter the cells. Both Neutral Red (Rashid *et al*., 1991) and potassium (Lote, 2000) can enter cells via passive diffusion. This common mechanism may explain why the intracellular Neutral Red concentration increased with potassium nitrate concentration; as potassium permeability increased, the Neutral Red dye also began to enter the cells at an increased rate.

Although this explanation does fit some of the findings of this study, others are still unexplained. It is possible that a high potassium load affects different cell types in a dissimilar manner, as the HepG2 cells did experience greater toxicity at higher potassium nitrate concentrations compared to the HEK293 cells. As mentioned above, liver cells may serve as a "sink" for excess potassium if intake is high, and could be exposed to high concentrations of potassium. Thus, it would be expected that liver cells may also have protective mechanisms which allow them to withstand high potassium concentrations. No consistent protective effect was observed in the HepG2 cells exposed to potassium nitrate. However, examination of the 24 hour KNO_3 Neutral Red assay in HepG2 cells does show a parabolic viability trend (i.e. increasing at low concentrations but decreasing at higher concentrations; see Figure 4.1). This may suggest that lower potassium concentrations initially have a protective effect on HepG2 cells, but this effect does not function at high potassium nitrate concentrations, or that the protective mechanisms are more active or effective in HEK293 cells due to the physiological role of the kidney.

Another important consideration involves the origin of the kidney cell line used in these experiments. The HEK293 cell line is derived from human embryonic kidney tissue, not an adult kidney; the line has also been transformed with sheared adenovirus 5 DNA. This may mean that HEK293 cells do not respond in the same way as an adult kidney cell culture, or an *in vivo* kidney. One investigation has found that HEK293 cells express many neuronal proteins, including several neurofilament subunits and α-internexin (Shaw *et al*., 2002). These authors argue that this finding indicates that HEK293 cells do not emulate typical kidney epithelial cells and should not be used for investigations examining the response of kidney cells. In light of these findings, the results obtained here should not be automatically assumed to mimic the behaviour of adult kidney cells or renal cells *in vivo*. The results of this study may reflect the unique properties of the HEK293 cell line, as opposed to those unique to human kidney cells *in vivo*. Additional testing with several other renal cell lines may clarify this issue.

Another potential explanation for the apparently increased toxicity of NH_4NO_3 compared to KNO_3 might be the way in which the ammonium ion affected the retention of the Neutral Red dye. As previously described, uncharged Neutral Red dye enters cells via passive diffusion, but is trapped in a charged form when it enters the low-pH environment of the lysosome (Rashid *et al*., 1991). When large amounts of ammonia and ammonium ions are present, such as during high-concentration NH_4NO_3 exposures, these may also enter the lysosome and increase the pH. An increase in lysosomal pH could result in less retention of Neutral Red dye, leading to a falsely low reading of viability in the affected cultures. Likewise, a decrease in ATP production in affected cells could prevent the operation of the ATP-dependent proton pumps surrounding the

lysosome (Rashid *et al*., 1991). This would result in an increase in lysosomal pH due to the cell's inability to maintain a low-pH environment, and would result in decreased Neutral Red trapping by the mechanism described above. Due to these potential confounding factors, it is recommended that these assays be repeated using another viability assay which is less sensitive to the presence of ammonia or ammonium ions.

However, it should be noted that interactions between the Neutral Red assay and the morphological changes which occur during cell death may also have contributed to the observed results. Morphological changes which accompany apoptotic cell death, which may be occurring in these cells, could also affect the entry and retention of Neutral Red dye. Normal apoptotic changes, such as decreasing cell volume, disruption of membrane potential, nuclear fragmentation, and decreased ATP production (Cooper, 2000) might also have prevented entry of the Neutral Red dye into still-viable cells. It should be noted, however, that cells exhibiting these changes may soon be rendered non-viable if apoptosis proceeds. Thus, the negative effect caused by this situation may be negligible.

5.1.1.3 Effect of nitrate exposure time on cell viability

Based on the results of the Neutral Red assays, increasing nitrate exposure time had a detrimental effect on HepG2 and HEK293 cell viability. This was especially evident in Figures 4.7 and 4.8, where the viability of HepG2 cells exposed to potassium or ammonium nitrate for 24, 48, or 72 hours was graphed. Viability at a single nitrate exposure concentration generally decreased as the exposure time increased; viability at 72 hours was usually lower than at 24 hours in cultures exposed to the same nitrate concentration. At lower nitrate concentrations, this observation was not always

consistent; cultures with longer exposure times occasionally had higher measures of viability than those with shorter exposure times. However, at mid- and high-range nitrate concentrations (>1500 mg/L), the relationship was generally valid. In Figure 4.8, which shows the viability of HepG2 cells exposed to ammonium nitrate for 24, 48, or 72 hours, the 72 hour viability appears higher than the 48 hour viability at some nitrate concentrations. The severity of decline over a relatively narrow range of nitrate concentrations could have obscured the differences between the 48- and 72 hour exposure groups; it is also important to observe that the trendlines for the two groups were very similar, though the 72 hour group showed the greatest decline. The EC_{50} values, seen in Table 4.1, confirm these observations; the EC_{50} values decreased as exposure times increased.

A similar situation may be seen in the HEK293 data. Although 48 hour nitrate exposures were not performed with HEK293 cells, comparison between the 24- and 72 hour exposure data shows that HEK293 cell viability also decreases with increasing exposure time. This may be seen in Figures 4.9 and 4.10, which demonstrate the effects of 24- and 72 hour exposures on cell viability in HEK293 cells after exposure to potassium or ammonium nitrate, respectively. As with HepG2 cells, viability was lower in cultures exposed to nitrate concentrations for longer periods of time. However, the HEK293 cells appeared to show more distinction between groups exposed to nitrate for different exposure times; no concentrations showed the 72 hour exposure group with greater viability than the 24 hour exposure group at the same nitrate concentration. The absence of a 48 hour exposure group in these cultures may have allowed greater resolution of the 24 and 48 hour datasets. Examination of the EC_{50} values for HEK293 viability assays

showed that the EC_{50} value decreased as exposure time increased (see Table 4.1); thus, increased exposure time resulted in increased toxicity to HEK293 cells. The 24 hour HEK293 KNO_3 exposure graph did not have an EC_{50} value, due to the upward slope of the graph; however, because the subsequent 72 hour KNO_3 exposure showed a downward-sloping trendline, it is reasonable to infer that this is a sign of increased toxicity with increasing exposure time.

Based on the available evidence, it appears that the differences in viability observed between HepG2 and HEK293 cells were caused by interactions between cell type and nitrate salt, but exposure time also appeared to play a role. Despite the possibility that protective adaptations to HEK293 cell function occurred with high concentrations of potassium nitrate in the 24 hour exposure, the 72 hour exposure showed a decline in cell viability. This may have been caused by the decline of the protective effect over time. One of the proposed adaptations to high potassium exposure for HEK293 cells is the increase in number and activity of Na^+/K^+/ATPase pumps. According to Kone (2004), the activity of the Na^+/K^+/ATPase pump consumes a substantial amount of energy. Activity of Na^+/K^+/ATPase is also thought to account for a significant portion of oxygen consumption in the kidney (Lote, 2000). It is possible that the cell is able to maintain this protective mechanism throughout the 24 hour exposure period, but that the increased stress gradually depletes the cell's energy resources and this mechanism fails by 72 hours of exposure. Likewise, the increased permeability to potassium (and subsequently to Neutral Red) which may have occurred in the HEK293 cells might have permitted maintenance of homeostasis through the 24 hour exposure, but began to fail by the 72 hour exposure. The increased permeability may have caused a breakdown in the cell

membrane, resulting in cell death and the reduction in viability observed in these experiments. This may also explain why the expression of Hsp70 did not appear to increase in cell cultures exposed to potassium nitrate; the Western blots were only performed on 24 hour exposure samples, which may not have allowed sufficient time for the full toxicity to develop.

5.1.1.4 Summary of findings for the effect of nitrate on cell viability

Based on the findings and hypotheses presented here, several conclusions may be made about the effects of nitrate on cell viability. A definite difference in cell viability was observed in HepG2 and HEK293 cells, particularly in the potassium nitrate exposure assays. This difference was likely not attributable solely to cell type, nitrate salt, and exposure time, but rather to a combination of these three factors. One hypothesis suggests that the reason for the apparent increase in viability seen in HEK293 cells in a 24 hour KNO_3 exposure was due to an increased permeability to Neutral Red dye as a consequence of activation of the unique potassium-handling mechanisms of the cells. This may have occurred in conjunction with a change in membrane potential brought about by high extracellular potassium concentrations, and the activation of potassium-handling mechanisms may have made the cells more resistant to nitrate toxicity. Conversely, the HepG2 cells may have been more sensitive because they do not possess the same homeostatic mechanisms as the HEK293 cells, possibly indicating a role of tissue specificity in nitrate toxicity. The increased toxicity observed in ammonium nitrate exposures could be due to the increased pH caused by the addition of NH_4NO_3 to the culture, or it might represent an additional stressor on both cell types which increased the susceptibility of both cell types to nitrate toxicity. Exposure time also

appeared to play a role, with both cell types showing decreasing viability with increasing nitrate exposure time. This may indicate a depletion of homeostatic mechanisms or processes over time. More research is needed to fully describe the effects of nitrate exposure on cell viability, but these findings represent an important first step in elucidating the mechanisms of nitrate toxicity.

5.1.2 Effects of nitrate on cell proliferation

As with the Neutral Red studies, some differences were observed between HepG2 and HEK293 cells when proliferation was observed after nitrate exposure. Like the viability studies, cell type, nitrate salt, and exposure time all affected the rate of cell proliferation seen in these experiments. Details of how these parameters may have affected the observed results are discussed in the following sections.

5.1.2.1 Effects of nitrate on cell proliferation in HepG2 vs. HEK293 cells

As described in section 4.1.2 and in Figures 4.11-4.14, it is evident that the HepG2 and HEK293 cells responded differently to nitrate exposure in these experiments. As evidenced by the EC_{50} values and the graphed proliferation values, the HepG2 cells were more sensitive in the 24 hour NH_4NO_3, 72 hour KNO_3, and 72 hour NH_4NO_3 assays. However, the HEK293 cells appeared more sensitive than the HepG2 cells in parts of the 24 hour KNO_3 assay, though both cell types had similar responses. For example, at the highest 24 hour KNO_3 concentration of 5000 mg/L, the HEK293 cells retained 43% of control proliferation while the HepG2 cells were reduced to 12% of control proliferation. At lower KNO_3 concentrations, however, HepG2 proliferation appeared to be higher than that of the HEK293 cells (see Figure 4.11).

In the other assays, although the HEK293 cells clearly retained a higher percentage of control proliferation than the HepG2 cells, the curve shape of the graphed data points were very similar. Upon examination of Figures 4.12-4.14, corresponding to the 24 hour NH_4NO_3, 72 hour KNO_3, and 72 hour NH_4NO_3 assays, the HepG2 and HEK293 data trendlines appear 'shifted' up or down on the y-axis relative to one another, suggesting that a similar mechanism of toxicity is occurring but affecting the two cell types to different degrees. The exact nature of this mechanism, however, remains unknown.

One puzzling finding is the response of the HEK293 cells in the 24 hour KNO_3 assay. In the 24 hour KNO_3 Neutral Red viability assay (see Figure 4.1 and section 5.1.1), the HEK293 cells experienced an apparent *increase* in cell viability with increasing KNO_3 concentrations. Although this finding may have been due to an interaction with the cell type, potassium cation, and Neutral Red dye, rather than an actual increase in cell viability, it is still puzzling to observe a decrease in cell proliferation in this assay, especially one that indicates that HEK293 cell proliferation is below that of HepG2 proliferation throughout the lower KNO_3 concentrations in the assay. However, careful examination of the data may explain this difference. In the 24 hour KNO_3 Neutral Red assay (see Figure 4.1), HepG2 viability is actually higher than HEK293 values until about the 2500 mg/L KNO_3 exposure concentration, but then begins to gradually decrease with higher KNO_3 concentrations. In the 24 hour BrdU proliferation assay (see Figure 4.11), the HEK293 proliferation percentage began to outstrip the HepG2 values at approximately the same KNO_3 concentration. Thus, the viability and proliferation data may both indicate a similar effect in these cells.

Both cell types also experienced initial 'spikes' in proliferation at the lowest nitrate concentrations tested in both potassium and ammonium nitrate exposures. In the HepG2 cells, spikes were seen in the 24 hour KNO_3, 24 hour NH_4NO_3, and 72 hour NH_4NO_3, although to a lesser extent in the latter assay. In the HEK293 cells, spikes were seen in 24 hour KNO_3, 24 hour NH_4NO_3 and 72 hour KNO_3 assays. The reason these spikes occurred is not completely clear, but several hypotheses are possible from what is known about the data. First, the spikes may be an artifact, generated by the extremely low nitrate concentrations used in the BrdU proliferation assay. With the exception of the 72 hour KNO_3 assay, all of the other proliferation values which exceeded the control value occurred at potassium or ammonium nitrate concentrations at or below 250 mg/L. These concentrations are below those used for the Neutral Red viability assays, and require several serial dilutions to prepare. Although great care was taken to ensure proper mixing of solutions when preparing the diluted samples, it is still possible that the resulting dilutions contained less nitrate than intended. Also, because the BrdU assay is performed in 96-well culture plates, as opposed to the larger flasks used in other cytotoxicity experiments, small differences in the volume of cell suspension or treatment solution could have had large effects on the proliferation values.

Conversely, if the effect observed was not due to an artifact, a hormetic response may be occurring. The definition of hormesis has been debated in the literature, but it generally refers to an initial increase in response at low toxicant concentrations, followed by a decreased response at higher concentrations. Hormetic responses to some toxicants have been observed in HepG2 cell culture lines (O'Brien *et al*., 2006; Calabrese, 2005), as well as one kidney tumour cell line (Calabrese, 2005). No literature on hormetic

responses is available for HEK293 cells. In these experiments, cell proliferation was found to be a sensitive indicator of hormesis (O'Brien *et al*., 2006), and a common compensatory mechanism for hepatocellular injury (Williams & Iatropoulos, 2002). Membrane permeability, however, was found to be the least sensitive toxicity/hormetic indicator in a study performed with HepG2 cells (O'Brien *et al*., 2006). If the results presented here indicate a true hormetic response, it may explain why an increase in proliferation was seen in the BrdU assay at low concentrations but the Neutral Red assay did not show a change in viability at similar concentrations. Because proliferation is a more sensitive marker of hormesis, the effect was visible at the lower concentrations of nitrate used in the study. However, the Neutral Red assays do not use nitrate concentrations below 500 mg/L and may not have been sensitive enough to detect a hormetic response. Alternately, a hormetic response may have manifested only in cell proliferation, not cell viability.

Several previous findings by other researchers may support the possibility of a hormetic effect in these experiments. The range of nitrate concentrations in the BrdU experiments which provoked the suspected hormetic effect are within the range cited to produce hormetic effects in other studies (Calabrese, 2005). Also, the proliferative spike appears to abate at longer exposure times, consistent with the findings of other studies which found that cytotoxicity increases over time and thus would result in a decreased hormetic response (O'Brien *et al*., 2006). Perhaps most interesting is the finding that methemoglobin, the product formed when nitrate (via nitrite) interacts with hemoglobin, is known to evoke a hormetic effect in NTU-Bw cells, another human hepatoma cell line (Calabrese, 2005). Nitric oxide, which may play a role in nitrate metabolism (see section

2.8), has also been shown to evoke a hormetic response in other human tumour cell lines (Calabrese, 2005). If substances linked to nitrate metabolism can provoke hormetic effects in human cell lines, it is possible this is also occurring in the current experiments.

Several findings of these assays, however, do not completely agree with what is known of hormetic effects in human cells. First, there is no available literature regarding hormetic effects in HEK293 cells, yet a hormetic-like effect has been observed in this cell line in these assays. This appears to be the result of limited exploration of the properties of HEK293 cells, but more research is required before conclusions can be made about hormetic properties in this cell line. Most existing studies which have examined hormesis in HepG2 cells have noticed an effect only after 72 hours of pre-incubation with the test substance (O'Brien *et al*., 2006; Calabrese, 2005); this finding disagrees with the current study, which observed an effect at 24 hours but a greatly decreased effect at 72 hours. The reasons for this discrepancy are unknown. Also, hormetic effects are thought to occur through a receptor-binding pathway (Williams & Iatropoulos, 2002; Calabrese, 2005), but it is unknown whether such a pathway is active during nitrate exposure and toxicity. The specific characteristics of the effect observed here are also somewhat different from descriptions of hormesis in the literature. In one study, most hormetic responses were measured at 150% of the control value (Calabrese, 2005); here, most of the observed responses were between slightly greater than 100% and 150%. Hormetic effects were not observed in every BrdU assay that was performed with HepG2 and HEK293 cells, and the nature of the effect varied somewhat from replicate plate to replicate plate. Unlike several studies cited in the literature (Calabrese, 2005), the suspected hormetic effect in this BrdU assay did not always follow a smooth

curved trendline; sometimes values fluctuated in a 'zig-zag' pattern both above and below the control value in consecutive nitrate concentrations. It is important to note, however, that the data presented for the BrdU assays are representative graphs, not pooled data as in the Neutral Red assay section. Thus, variability between BrdU replicates is not shown on the graphs, and the possibility for error may be increased. The reasons for these differences are unknown, as these experiments were not designed to examine these questions. However, based on the available information, further studies into the potential role of hormesis in the response of HepG2 and HEK293 cells to nitrate exposure would be beneficial.

5.1.2.2 Effects of potassium nitrate vs. ammonium nitrate on cell proliferation

An examination of the data obtained from the BrdU proliferation assays has revealed differences in cell response between the two nitrate salts used in these experiments. As seen by Table 4.2, EC_{50} values were lower for NH_4NO_3 exposures than KNO_3 exposures in the same exposure time, suggesting that NH_4NO_3 was more toxic to both HepG2 and HEK293 cells in these experiments. Also, examination of Figures 4.11-4.14 show that the shape and slope of ammonium nitrate exposure graphs is more depressed than the potassium nitrate exposure graphs; this indicates greater toxicity to the exposed cells. Conversely, KNO_3 exposure graphs appeared to have more data points where exposed cell proliferation exceeded control group proliferation. This was especially evident in the 72 hour HEK293 KNO_3 assay, where 9 of 13 KNO_3 concentrations tested produced proliferation values exceeding those of the control group (see Figure 4.13). Because the 24- and 72 hour KNO_3 assays show some interesting differences, these will be discussed in section 5.1.2.3.

The reasons for the apparent salt-specific effects of nitrate on cell proliferation may be similar to those described in the Neutral Red assay discussion (section 5.1.1). High nitrate concentrations represent a stressor for both cell lines, and the homeostatic mechanisms probably induced with high nitrate exposure likely require significant cellular resources to maintain. The drain in available energy, combined with the stress, results in a lower rate of cell proliferation because the cell does not have enough resources to allocate to mitosis. The HEK293 cells may have appeared less affected in the KNO_3 assays because the physiological properties of kidney cells may confer a greater resistance to high potassium concentrations (see section 5.1.1.1). However, the confounding influence of increased membrane permeability, as discussed in the Neutral Red assay section, is likely not a factor in the BrdU ELISA assay because the BrdU compound must be physically incorporated into the DNA to be measured.

As with the Neutral Red assays, the cation paired with the nitrate anion appears to modify the toxicity to the cell lines used in these experiments. Because of the very high concentrations of nitrate salts used in these experiments, the toxicity of the cation itself likely contributes significantly to the overall toxicity observed. As in the Neutral Red assays, ammonium nitrate appears to have greater toxicity for both HepG2 and HEK293 cells compared to potassium nitrate under these experimental conditions. However, the differences between NH_4NO_3 and KNO_3 toxicity are not as pronounced in the BrdU proliferation assay compared to the Neutral Red viability assay. The reason for this finding is unknown. Possible explanations for the greater NH_4NO_3 toxicity responses include an increase in pH, or a possible additional stressor unique to the ammonium ion which increased cell susceptibility to nitrate toxicity (see sections 5.1.1.1 and 5.1.1.2 for

further discussion). More research is required to fully understand why and how the different salts of nitrate induce differing degrees of toxicity.

The proposed hormetic response appeared to occur more frequently in the potassium nitrate assays, although a substantial spike in proliferation was also noted in the 24 hour NH_4NO_3 assay. The reason for this finding is unclear, but might relate to the relatively lower toxicity of KNO_3 as opposed to NH_4NO_3. The reduced degree of toxicity might allow the hormetic effect to become more noticeable, while a high degree of toxicity might simply mask any initial increase in proliferation with a rapid and severe decrease. Conceivably, a more toxic substance might require more minute doses of toxicant to evoke hormesis (assuming it occurs at all) compared to a less toxic substance, and thus the hormetic range might fall below concentrations used in these assays or below the analytical limits of detection. However, caution must be used when interpreting these findings; the involvement of a hormetic mechanism in nitrate toxicity is a hypothesis, not a confirmed finding.

In summary, the choice of nitrate salt did appear to have an effect on cell proliferation in both HepG2 and HEK293 cells under these experimental conditions, though this effect appeared less prominent than in the Neutral Red assay. Potassium nitrate appeared less toxic to both HepG2 and HEK293 cells than ammonium nitrate, though both substances evoked decreases in cell proliferation compared to control. The physiological properties of each cell type tested may have modulated the nitrate toxicity observed. Differences in pH or salt-specific properties may have also affected toxicity. The proposed hormetic effect noted in section 5.1.2.1 may also have been affected by the choice of nitrate salt,

with more data points showing elevated proliferation compared to control in the KNO_3 assays compared to the NH_4NO_3 assays. This may be due to the nature of the hormetic response, but further studies are required to better characterize this effect.

5.1.2.3 Effects of exposure time on cell proliferation

From the results of the BrdU ELISA assays, increasing exposure time to nitrate appeared to have a negative effect on cell proliferation in both HepG2 and HEK293 cell lines under most circumstances. This finding is similar to the Neutral Red assay results, which found increasing toxicity with increasing exposure time. However, not all assays followed this pattern; some important differences were found and are described below.

Exposure time had a substantial effect on proliferation in both HepG2 and HEK293 cells in NH_4NO_3 assays. Both cell types experienced a profound decrease in cell proliferation compared to the control group with increasing time, as seen in Figures 4.12 and 4.14. In the 24 hour NH_4NO_3 assay, both cell types experienced substantial declines in cell proliferation but did not reach negative values compared to the control group. However, negative proliferative values (as calculated after subtraction of the blank reading) compared to the control group were noted for both cell types in the 72 hour NH_4NO_3 assay, with 8 of the 13 concentrations tested in HepG2 cells producing negative values. A comparison of the EC_{50} values for the NH_4NO_3 exposures reinforces the concept that increased exposure time resulted in increased toxicity; the values were 734 mg/L in HepG2 cells and 1442 mg/L in HEK293 cells in the 24 hour exposure, but approximately 2.5 mg/L in HepG2 cells and 471 mg/L in HEK293 cells in the 72 hour exposure (see Table 4.2).

However, the effect of exposure time on cell proliferation in KNO_3 assays was less consistent between the two cell types. In HepG2 cells, increasing exposure time resulted in decreased cell proliferation, as seen by Figures 4.11 and 4.13. Although both 24 and 72 hour assays evoked large declines in HepG2 proliferation compared to the control group, the 72 hour assay evoked a sharp decline in proliferation at much lower concentrations and resulted in a negative proliferation value at the highest KNO_3 concentration tested (see Figure 4.13). The EC_{50} values for 24 and 72 hour KNO_3 exposures in HepG2 cells support this finding; the values were 1943 mg/L in the 24 hour KNO_3 assay but 730 mg/L in the 72 hour KNO_3 assay. This finding agrees with the Neutral Red assay data, which found that increasing exposure time resulted in decreased cell viability in HepG2 cells (see section 5.1.1.2).

The HEK293 cells demonstrated a different and unexpected response to potassium nitrate with increasing exposure time. In contrast with the expected findings, the 24 hour KNO_3 exposure appeared to evoke a greater decrease in HEK293 cell proliferation compared to the control group than the 72 hour KNO_3 exposure (see Figures 4.11 and 4.13). The 24 hour HEK293 KNO_3 assay showed a small initial increase in proliferation before a decline in proliferation compared to the control group, but the 72 hour KNO_3 exposure resulted in proliferation values in excess of the control group for the majority of nitrate concentrations tested. The EC_{50} values also demonstrate this unexpected finding, with the value for the 24 hour KNO_3 exposure (1737 mg/L) less than the value for the 72 hour KNO_3 exposure (3631 mg/L) in HEK293 cells. These findings do not agree with the Neutral Red assay results, which found that the toxic effects of potassium nitrate on HEK293 cell viability increased with increasing exposure time.

The reasons for these contradictory findings are unknown. The explanation for the sustained increase in proliferation compared to control in the 72 hour KNO_3 assay does not appear to involve a hormetic response, as the increase appears sustained over a wide range of KNO_3 concentrations. A typical hormetic response involves an increase in response at low concentrations and then a decreased response at higher concentrations; this pattern does not appear to be replicated in the 72 hour KNO_3 assay. The elevated toxicity observed in the 24 hour KNO_3 assay also does not appear to have an apparent explanation. It is possible that cell proliferation was depressed during the 24 hour period because energy and resources were being redirected to homeostatic mechanisms. However, it does not make sense that proliferation at the 72 hour exposure mark would then outstrip the control group, especially because the Neutral Red assay indicated that potassium nitrate decreased cell viability in HEK293 cells during the same exposure period (see section 5.1.1). If homeostasis had been reached, then it might be conceivable that proliferation would return to control levels, but it does not appear that this is occurring in this group. Considering these contradictory findings, there is a possibility that this finding was an artifact. Further study is required to determine what is occurring in this situation.

5.1.2.4 Summary of findings for the effect of nitrate on cell proliferation

In summary, it appears that nitrate exposure generally had a negative effect on HepG2 and HEK293 cell proliferation under these experimental conditions. As with the Neutral Red viability assay, cell type, nitrate salt, and exposure time appear to interact to cause toxicity. The HepG2 cells appeared to be more sensitive than HEK293 cells in the majority of assays conducted, although the shape and nature of the trendlines for each

data graph were similar. It is possible that the effects of nitrate on HepG2 and HEK293 cell proliferation follows a similar mechanism, due to the similarities between the responses noted. Initial spikes in proliferation were observed for some of the studies, and may indicate a possible hormetic effect. Previous literature indicates that some of the results of these studies correspond with a classical hormetic response. However, not all findings are explained by this model, and the involvement of hormesis in nitrate toxicity should be regarded as a hypothesis, not an empirical finding. Ammonium nitrate appeared to induce a greater decline in proliferation in both cell types compared to potassium nitrate. Exposure time appeared to negatively affect HepG2 cell proliferation but had a contradictory effect on the KNO_3 HEK293 exposures. The reasons for this are currently unknown, but further study should further elucidate the mechanisms behind these intriguing findings.

5.1.3 Comparison of the effects of nitrate on cell viability and cell proliferation

Generally, the Neutral Red viability assay and the BrdU ELISA assay showed that nitrate exposure has detrimental effects on the HepG2 and HEK293 cell lines under these experimental conditions. Although some exceptions existed, the data obtained from the two studies were in good general agreement regarding the effects of nitrate on human cell lines.

The BrdU ELISA assay for cell proliferation appeared to be the most sensitive measurement of the toxic effects of nitrate compared to the Neutral Red assay. A decrease in cell proliferation was observed in the BrdU ELISA assay when little or no reduction in cell viability was apparent in the Neutral Red assay, for example in the 24

and 72 hour HEK293 KNO_3 assays. The BrdU ELISA EC_{50} values were consistently lower than those of the Neutral Red assay, indicating that discernable toxicity was likely occurring at lower nitrate concentrations. The shapes of the trendlines and data points for the BrdU ELISA assay graphs also appeared to be more severe than those of the Neutral Red assay graphs. The BrdU ELISA trendlines tended to follow an exponential or quadratic polynomial pattern, while the Neutral Red trendlines tended to be more linear. However, exceptions did occur in both groups.

For the majority of experiments with both assays, the HepG2 cells appeared to be more sensitive to nitrate than the HEK293 cells. This was particularly noticeable in the KNO_3 Neutral Red exposures, where HepG2 cell viability decreased while HEK293 viability increased (24 hour KNO_3 assay; see Figure 4.1) or remained nearly unchanged (72 hour KNO_3 assay; see Figure 4.5). The NH_4NO_3 Neutral Red assays, however, demonstrated that HEK293 cells were more sensitive to NH_4NO_3 in the 24 hour exposure (see Figure 4.2), but not the 72 hour exposure (see Figure 4.6), where they were slightly less sensitive than the HepG2 cells. In the BrdU assays, HepG2 cells appeared more sensitive than HEK293 cells in all of the assays except the 24 hour KNO_3 exposure, where HepG2 cells appeared less sensitive at low nitrate concentrations (possibly due to a hormetic effect; see section 5.1.2.1) but more sensitive at higher concentrations compared to the HEK293 cells. When all assays are considered, HepG2 cells appear to be more sensitive to negative effects of nitrate on cell viability and proliferation compared to HEK293 cells under these experimental conditions.

Results from the two assays also suggest that the cation paired with nitrate has an effect on the observed toxicity. In both the Neutral Red and BrdU ELISA assays, the EC_{50} values for NH_4NO_3 were lower than those for KNO_3, indicating greater toxicity at lower concentrations. Assays performed with NH_4NO_3 showed more severe declines in cell viability and proliferation compared to KNO_3, as evidenced by the steepness and shape of the data trendlines in the graphed assays. All of the assays conducted with HEK293 cells exposed to KNO_3 produced unexpected results, indicating that an interaction may have occurred between the cell type and the potassium nitrate salt. Exposure of HEK293 cells to potassium nitrate produced higher-than-expected viability in the Neutral Red assay, but paradoxically increased proliferation at the 72 hour exposure period compared to the 24 hour exposure period in the BrdU ELISA assay. The reasons for this are unknown, but may include an activation of physiological potassium-handling mechanisms unique to the HEK293 cells, or a hormetic response. When all data are considered, salt-specific toxicity was likely a factor in both the Neutral Red and BrdU ELISA assays, with ammonium nitrate demonstrating greater toxicity to both cell types compared to potassium nitrate. In both assays, potassium nitrate appeared to interact with cell type to produce unexpected results. The mechanisms of this interaction are unclear but may be solved with further research.

The effect of exposure time on resulting nitrate toxicity appeared to be largely consistent between the two assays. Increased exposure time resulted in increased toxicity, as measured by decreased viability in the Neutral Red assay and decreased proliferation in the BrdU ELISA assay. All of the Neutral Red assays performed showed an increase in toxicity with lengthened exposure time. Most of the BrdU ELISA assays also

demonstrated an inverse relationship with exposure time and cell proliferation compared to control. However, the 24- and 72 hour HEK293 KNO_3 assays demonstrated the opposite trend; proliferation was elevated in the 72 hour exposure and reduced in the 24 hour exposure (see Figures 4.11 and 4.13). This appears to contradict the findings of the Neutral Red assay, and the mechanism or reason for this finding is currently unexplained. Because of the contrasts with the Neutral Red data and what is known about nitrate toxicity, the finding may be an artifact. Despite this difference, it appears that increasing exposure time generally results in increasing toxicity for both the Neutral Red and BrdU ELISA assays conducted with HepG2 or HEK293 cells under these experimental conditions.

As discussed in section 5.1.2.1, a possible explanation for the initial spikes observed in some of the BrdU ELISA assays could be the presence of a hormetic effect. However, such an effect was not observed in the Neutral Red assays. This was potentially due to the increased sensitivity of proliferation as a hormetic marker, and the use of lower concentrations in the BrdU ELISA assay, allowing visualization of the effect. Conversely, the increase in viability seen in HEK293 cells with increasing KNO_3 concentration in the 24 hour Neutral Red assay is hypothesized to occur though unique physiological potassium-handling homeostatic mechanisms, including an increase in membrane permeability. This did not likely have an effect on the results of the BrdU ELISA assay, since the BrdU compound must be incorporated into the DNA in order to be detected. These differing hypotheses may explain why unique and unexpected results occurred in each assay.

In conclusion, although the Neutral Red and BrdU ELISA assays measure different cytotoxic parameters, they appear to be in good agreement regarding the toxic effects of nitrate exposure on the HepG2 and HEK293 cell lines under these experimental conditions. Good correlations were found for the modifying effects of cell type specificity, nitrate salt toxicity, and exposure time. Although the effects of KNO_3 on HEK293 cells appeared to differ between the two assays, the other findings were in general agreement. This represents an important finding, as agreement between the Neutral Red and BrdU ELISA assays indicate that nitrate exposure does induce toxicity in these human cell lines and has the potential to affect two important intracellular processes.

5.2 Discussion of control and alternate salt experiments

5.2.1 Media control experiments

As described in section 4.2.1.1, significant variability between media control flasks was observed in the 24 hour (but not 72 hour) media exposure groups. These assays were performed using the Neutral Red assay protocol, as described in section 3.5. This finding raises the possibility that some of the variation observed in the experimental assays may not have been due to genuine treatment responses, but rather to intra-experiment variability.

Several potential scenarios may explain the observed variation. One of these was the Neutral Red protocol itself; several issues associated with sample processing may have increased variability of the results. As described in section 3.5, sample flasks were subjected to several washing steps with phosphate-buffered saline (PBS). If the PBS

solution was allowed to remain in contact with the adherent cells for too long, cell detachment may have occurred. This finding was particularly significant for HEK293 cell cultures, who displayed reduced adherence to the culture plates and were thus more susceptible to detachment. Processing large numbers of culture flasks at one time increased the amount of contact time PBS solution had with the cells, as a longer period elapsed between application and removal of the solution. Also, cultures with a greater proportion of viable cells at the end of the experiment may also be more susceptible to experimental error. If great care was not taken to minimize movement of the culture flask during the washing steps, the adherent cells may have peeled off in 'sheets' which were not analyzed or included in the experiment. This may have greatly increased error, and was more prevalent in HEK293 cultures due to their reduced culture plate adherence.

Another possible explanation for the degree of observed variation could be biological variation within the cell cultures. Although as much care as possible was taken to ensure all cells were subjected to the same culture conditions and experimental manipulations, it is possible that not all cells within each flask responded in the same way. Repeated passaging of cultures may invoke changes in gene expression (Wilkening & Bader, 2003), which would increase the effects of biological variation. However, because all of the cells in each media control experiment were of the same passage, this effect was likely minimal.

Although the above scenarios are plausible explanations of the observed increase in variation, modifications to the experimental protocol were made to minimize their

effects. To counteract the negative effects of PBS washing, the solution was not allowed to contact the cells for longer than necessary, and experiments were staggered for processing in small batches rather than large ones. As described in section 3.5.1, modifications to the Neutral Red protocol were made for the HEK293 cultures to prevent cell loss through detachment. If flasks experienced significant loss of cells in 'sheets', the affected flask was discarded. Adjustment for biological variation was achieved by using multiple repetitions of each experiment to determine the final response – either by pooling data (Neutral Red assay), or using representative data (BrdU ELISA). The number of passages for each culture used in these experiments was kept to a minimum to prevent changes in gene expression (see section 3.2).

Due to the precautions observed, it appears unlikely that any variation observed in the media control experiments greatly affected the nitrate experimental groups. Potentially confounding factors such as experimental protocol and biological variation were countered through the use of modified experimental procedures and multiple repetitions of each experiment. The passage history of each cell culture was considered in order to minimize changes in gene expression or biological variation. Although these steps probably did not counteract all intra-experiment variability, it was likely reduced to an acceptable level. Thus, all significant differences observed in the experimental groups were likely due to actual treatment responses rather than experimental error or biological variation.

5.2.2 48 hour HepG2 alternate salt exposure – Neutral Red

The results of this assay were somewhat surprising, as potassium acetate was not originally expected to induce the greatest toxicity in HepG2 cells. From the results of the Neutral Red (see section 5.1.1) and BrdU ELISA (see section 5.1.2) assays, the ammonium salt of acetate would be expected to be more toxic than the potassium salt. In the experimental Neutral Red assays with KNO_3 and NH_4NO_3, ammonium nitrate consistently induced greater toxicity than potassium nitrate. Because the viability reduction curves for CH_3COOK and CH_3COONH_4 display similar shapes yet different magnitudes (see Figure 4.17), the choice of cation may also have influenced toxicity in this experiment, though obviously not in the same manner as the nitrate experimental assays. It is possible that the coupling of these cations with acetate may have caused different interactions with the HepG2 cells, and thus a different pattern of toxicity than what was observed in the nitrate assays. The toxicity of the acetate anion may have overshadowed the toxicity of the individual cations, and thus produced a unique response. Or, the acetate ion itself may have interacted with the liver cells to produce the observed toxic effect. Acetate was historically used as a buffer in dialysate solution, as it may be converted into bicarbonate by the liver (DailyMed, 2006); however, buildup of acetate ions *in vivo* due to slow conversion rates resulted in "adverse metabolic and hemodynamic effects" (Schulman & Himmelfarb, 2004). It is possible that the same situation is occurring here, with toxicity resulting to the HepG2 cells because of a buildup of acetate anions.

The comparatively low toxicity of sodium nitrate was also surprising, as other nitrate salts have shown considerable toxicity to HepG2 cells in the experimental Neutral Red

assays (see section 5.1.1). However, as with the CH_3COOK and CH_3COONH_4 assays described above, the toxicity of the acetate anion is likely distorting the perception of $NaNO_3$ toxicity when all three responses are plotted on the same graph. The calculated EC_{50} value for the $NaNO_3$ assay, 5820 mg/L, is greater than the 48h KNO_3 EC_{50} value of 3862 mg/L. Thus, the sodium salt of nitrate appears to have a reduced toxicity to HepG2 cells in the Neutral Red assay compared to the potassium nitrate salt. It is possible that the sodium cation modifies the toxicity of the nitrate, as hypothesized for both the potassium and ammonium salts (see section 5.1.1).

If so, the relative toxicities of each nitrate salt in HepG2 cells from the results of the Neutral Red experiments appear to be $Na^+ < K^+ < NH_4^+$. A comparison of this ranking with *in vivo* LD_{50} data yielded interesting results. For potassium and ammonium nitrate, rat oral LD_{50} values were consistent between a variety of sources; these corresponded to 3750 mg/kg for KNO_3 (Sigma-Aldrich, 2006a) and 2217 mg/kg for NH_4NO_3 (Sigma-Aldrich, 2006b). However, the LD_{50} values available for oral administration of $NaNO_3$ to rats are widely variable, including values of 4860-9000 mg/kg (WHO, 1996) to 3236 mg/kg (WHO, 1965) to 1267mg/kg (Sigma-Aldrich, 2006c). This makes the relative comparison between the salts of nitrate used in these experiments difficult, as these three values indicate $NaNO_3$ is the least, middle, and most toxic of the three nitrate salts, respectively. If only the oral rat LD_{50} values used on Sigma-Aldrich MSDS sheets (2006) are considered, the acute oral toxicity ranking of each nitrate salt is $K^+ < NH_4^+ < Na^+$, which is different from the results of this study.

A comparison of the other alternate salt LD_{50} values yielded additional interesting results. The rat oral LD_{50} value for CH_3COOK was 3250mg/kg (Sigma-Aldrich, 2007a), which would theoretically place potassium acetate as slightly more toxic than potassium nitrate and slightly less toxic than ammonium nitrate on the relative scale. However, the EC_{50} values obtained for CH_3COOK in this study were much lower than those for potassium nitrate in the same exposure period of 48 hours (CH_3COOK EC_{50} = 1773 mg/L; KNO_3 EC_{50} = 3862 mg/L), and slightly higher than those for ammonium nitrate (NH_4NO_3 EC_{50} = 1686 mg/L). A direct comparison for ammonium acetate is not possible because only rat intraperitoneal LD_{50} values are available for this substance. Thus, according to the published LD_{50} values, the relative toxicity scale is KNO_3 < CH_3COOK < NH_4NO_3 < $NaNO_3$, but the EC_{50} values generated by the current study result in a ranking of $NaNO_3$ < CH_3COONH_4 < KNO_3 < CH_3COOK < NH_4NO_3. It is interesting to note that, with the exception of the sodium nitrate ranking (and the absence of a rat oral LD_{50} value for CH_3COONH_4), all other toxicants are given the same relative rank from the *in vivo* and *in vitro* data. It is important to remember, however, that a decrease in cell viability seen *in vitro* is not directly comparable to the death of an experimental animal *in vivo*; the rankings are provided for theoretical purposes only. The reason for the relative differences in toxicity between the current *in vitro* study and the *in vivo* studies used to produce the LD_{50} values are unclear, but may involve the interaction of other body systems not present in cell cultures.

Another surprising finding was the presence of an initial spike in cell viability compared to the control group in some of the HepG2 alternate salt Neutral Red assays. All salts tested produced at least one measurement of viability greater than the control group at

low concentrations of nitrate. The CH_3COONH_4 and $NaNO_3$ groups contained four and five concentrations of nitrate, respectively, which produced these excessive viability measurements. These corresponded to concentrations of 3 mg/L, 45 mg/L, 150 mg/L, and 500 mg/L in both salts, plus the 1000 mg/L concentration in $NaNO_3$ alone. It is important to note that the concentrations of 3 mg/L, 45 mg/L, and 150 mg/L were not used in the KNO_3 and NH_4NO_3 Neutral Red assays. The reason for these elevated measurements is unclear. It is possible that this may correspond to a hormetic effect, as hypothesized for the BrdU ELISA data (see section 5.1.2). However, it is surprising to see a potential hormetic effect in the viability assay because membrane permeability, potentially the cause of increased Neutral Red dye retention (see section 5.1.1), has been found to be one of the least sensitive indicators for a hormetic effect (O'Brien *et al*., 2006). Because Neutral Red enters cells via passive diffusion, an increase in membrane permeability was hypothesized to occur in situations where Neutral Red uptake was increased; however, this hypothesis is theoretical and was not empirically tested in the course of these experiments. Also, the largest observed values were not substantially above the control group values; most ranged from slightly over 100% to 109% of control group viability. According to Calabrese (2005), hormetic responses often occur at 150% of the control value; none of the responses in the 48 hour HepG2 alternate salts Neutral Red assay were near this value. The previous potentially hormetic responses observed in the nitrate-treated BrdU ELISA assays (see section 5.1.2.1) were also much higher than these responses, with values between just over 100% to 150% of control group response. None of the experimental values in this assay were statistically significantly different from control group values. This may indicate that the observed response is either a very weak hormetic response, which is possible because membrane permeability is not a

sensitive indicator of hormesis, or that a hormetic response is not occurring and the results may be attributed to biological variation.

To summarize, the results of the 48 hour HepG2 alternate salt Neutral Red assays were somewhat unexpected. Potassium acetate evoked the greatest decrease in cell viability compared to the control group, followed by ammonium acetate and sodium nitrate. This may have been due to an interaction between acetate and the HepG2 cells which resulted in greater toxicity. An examination of the EC_{50} values for the alternate salts and nitrate salts tested in HepG2 cells using the Neutral Red assay revealed that the relative ranking of toxicity for each substance was $NaNO_3 < CH_3COONH_4 < KNO_3 < CH_3COOK < NH_4NO_3$. This is slightly different from the relative rankings using published rat oral LD_{50} values, which place the ranking at $KNO_3 < CH_3COOK < NH_4NO_3 < NaNO_3$. No comparison could be made for CH_3COONH_4 due to the absence of a rat oral LD_{50} value. A possible hormetic response was observed in the CH_3COONH_4 and $NaNO_3$ groups, but due to the weak quality of the response it may have been an artifact. The 48 hour HepG2 alternate salt Neutral Red assays provided valuable information about the effects of anion/cation interactions, a potential hormetic response, and the relative rankings of *in vitro* toxicity compared to *in vivo* toxicity.

5.2.3 48 hour HEK293 alternate salt exposure – Neutral Red

As with the 48 hour HepG2 alternate salt Neutral Red assays (see section 5.2.2), the relative toxicities of each alternate salt were somewhat surprising. The apparently very low toxicity of $NaNO_3$ was again unexpected, and so was the strong toxic effect of CH_3COONH_4, though to a lesser extent. Like the findings of the HepG2 alternate salt

Neutral Red assays, the choice of cation and anion did appear to have an effect on the resulting toxicity, especially in the case of sodium nitrate. A comparison of the trendlines for the HEK293 $NaNO_3$ group in this assay and the 72 hour HEK293 KNO_3 Neutral Red assay shows a similar slope and overall shape, suggesting an effect of a similar magnitude exerted on both cell types by the nitrate exposure. A comparison of the EC_{50} values at the same timepoint is not possible because a 48 hour KNO_3 exposure was not performed on the HEK293 cells. The extremely low toxicity observed in both the KNO_3 and $NaNO_3$ groups suggests that a common mechanism may be involved in the apparent resistance to the toxic effects of nitrate. In section 5.1.1.2, it was hypothesized that the HEK293 cells may have possessed unique potassium-handling mechanisms which conferred increased resistance to cell damage due to the physiological role of the kidney in potassium handling. A similar situation may be occurring with the sodium salt of nitrate, as the maintenance of sodium homeostasis is also part of the physiological function of the kidney (Lote, 2000). Sodium and potassium flow in the kidney are tightly linked, with many processes, such as the sodium-potassium pump (Na^+/K^+/ATPase) dependent on the concentrations of both cations (Malnic *et al*., 2004). It is possible that exposure to sodium nitrate induced the same homeostatic mechanisms as potassium nitrate, leading to a protective effect. A full discussion of the interactions between sodium and potassium in the kidney is beyond the scope of this thesis, but the basic knowledge that interactions exist may explain why HEK293 cells appeared more resistant to the potassium and sodium salts of nitrate.

The EC_{50} value for a 48 hour HEK293 CH_3COONH_4 exposure in this assay (1328 mg/L) was lower than any of the EC_{50} values produced in the HEK293 nitrate experimental

Neutral Red assays; the closest EC_{50} value is that of the 72 hour HEK293 NH_4NO_3 exposure (1557 mg/L, see Table 4.1). The substantial toxicity observed in the 48 hour HEK293 CH_3COONH_4 alternate salt exposures may be due to the toxic effects of acetate, as hypothesized in section 5.2.2. An interaction between the acetate ion and the HEK293 cells (and possibly also the ammonium cation) may have resulted in greater toxicity than was observed in the nitrate experimental assays. Unfortunately, no 48 hour HEK293 Neutral Red assays with CH_3COOK were performed, so a comparison between the two acetate salts is not possible.

Examination of Figure 4.18, where the results of the 48 hour HEK293 alternate salt Neutral Red experiments are graphed, reveals the absence of an initial spike in cell proliferation. Conversely, a proposed hormetic effect was observed in the 48 hour HepG2 alternate salt Neutral Red assays (see section 5.2.2). However, the reason for this observation stems from the use of different concentration ranges, not a tissue-specific response. For the HepG2 alternate salt Neutral Red assays, alternate salt concentrations of 3, 45, and 150 mg/L were added to the previously established range of 500-5000 mg/L used in the nitrate-exposure Neutral Red experiments. Unfortunately, only a range of 500-5000 mg/L was used in the HEK293 alternate salt Neutral Red assays, as the decision was made to include the additional concentrations only after these assays were finished. This is unfortunate because it precludes a comparison between the two cell types to determine if a hormesis-like response was occurring. However, due to the weak nature of the proposed hormetic response in the 48 hour HepG2 alternate salt Neutral Red assays, it is possible that the observed effect was an artifact instead of an actual response. Repetition of the 48 hour HEK293 alternate salt Neutral Red assay with the

expanded concentration range and the addition of a CH_3COOK group would provide valuable data about the possibility of a hormetic response and also allow a full comparison between the HepG2 and HEK293 alternate salt Neutral Red data.

From the results of the HEK293 48 hour alternate salt and nitrate Neutral Red assays, the relative toxicity rankings for the tested substances (based on EC_{50} values) are as follows: $KNO_3 < NaNO_3 < NH_4NO_3 < CH_3COONH_4$. It is important to note that the EC_{50} values used are not all from the same timepoint; 48 hour Neutral Red exposures were not performed in HEK293 cells for KNO_3 and NH_4NO_3, so the 72 hour EC_{50} values were used instead. The relative toxicity scale of these substances based on published rat oral LD_{50} values is $KNO_3 < NH_4NO_3 < NaNO_3$, as described in section 5.2.2. Ammonium acetate is not included in this ranking because no rat oral LD_{50} value exists. The results obtained in this study, with the exception of the sodium nitrate data, place the tested compounds in the same relative order of toxicity as the published rat oral LD_{50} values. Again, however, it must be reiterated that the two endpoints of *in vitro* cell death and *in vivo* experimental rat death are not equivalent, and that this ranking is for theoretical purposes only.

In summary, the results of the 48 hour HEK293 alternate salt Neutral Red assay showed that ammonium acetate was much more toxic to HEK293 cells than sodium nitrate. Like the HepG2 Neutral Red assays conducted with the same salts (see section 5.2.2), this may have been due to an interaction between the acetate anion and the HEK293 cells which resulted in greater toxicity. Because the responses of the HEK293 cells exposed to KNO_3 and $NaNO_3$ were very similar, it is possible that the nitrate salts affected the

toxic response in the same way. Similar homeostatic mechanisms may have been involved in the reduction of toxicity in the sodium and potassium nitrate salts; this is supported by the close relationship between sodium and potassium regulation in the kidney. However, this is only a hypothesis and experiments designed to test this theory are required. No hormetic-like effect was observed in these data sets; however, this likely stems from the different concentration range used in these experiments (compared to the HepG2 alternate salt Neutral Red experiments) rather than the actual absence of a hormetic effect. The relative ranking of toxicity for HEK293 cells in these experiments, based on the EC_{50} values obtained, was $KNO_3 < NaNO_3 < NH_4NO_3 < CH_3COONH_4$. This differed from the ranking scheme using rat oral LD_{50} data, which placed the relative toxicities as follows: $KNO_3 < NH_4NO_3 < NaNO_3$. Again, no comparison could be made with CH_3COONH_4 since no rat oral LD_{50} value exists. From these assays, information was gained about the effects of anion/cation toxicity on HEK293 cells, and the relative ranking of the nitrate and alternate salts used for *in vitro* testing. Ideally, more experiments are required to fully compare the effects of alternate salts on HepG2 and HEK293 cells, but these experiments have provided valuable preliminary data which may be used to guide further explorations.

5.2.4 48 hour HepG2 alternate salt exposure – BrdU ELISA

Upon examination of Figure 4.19, where the results of the HepG2 alternate salt ELISA assays are graphed, it is evident that the acetate salts produced much greater reductions in HepG2 proliferation than sodium nitrate. As hypothesized during the discussion of the Neutral Red alternate salts assays, it is possible that acetate interacts with HepG2 cells in a way that produces greater toxicity. Both acetate salts evoked a massive decline in cell

proliferation compared to control; at 5000 mg/L, the CH_3COOK group response was only 1% of the control response, and the CH_3COONH_4 group response was only 7% of the control group. By contrast, the $NaNO_3$ group retained 60% of control group proliferation at the same concentration. Perhaps more interesting is the finding that CH_3COONH_4 appeared to evoke a greater toxic response than CH_3COOK. In the HepG2 Neutral Red alternate salts assays (see section 5.2.2), CH_3COOK was shown to have a greater toxic effect on HepG2 cells compared to CH_3COONH_4. In the current proliferation assays, however, this effect appears to be reversed. The reasons for this difference are unknown; however, from these results it appears that CH_3COONH_4 may affect the proliferative capability of the HepG2 cells differently than it affects cell viability. The mechanism of this effect is unknown, but deserves further study.

As noted above, all of the alternate salts tested in this experiment produced a proliferation value greater than that of the control in at least one concentration tested. The CH_3COOK study had 7 of 13 concentrations evoke a response larger than the control group, with one statistically significant increase at 45 mg/L and a range of responses from 101% - 140% of control. The CH_3COONH_4 study had 5 of 13 concentrations evoke cell proliferation greater than that of the control group, with three statistically significant increases at 1 µg/L, 3 mg/L, and 250 mg/L and a response range of 100% - 160%. Finally, the $NaNO_3$ study had 10 of 13 concentrations evoke higher-than-control proliferation, with two statistically significant increases at 45 mg/L and 250 mg/L and a response range of 105% - 177%. These results are indicative of a potential hormetic response, as described in section 5.1.2.2. Evidence supporting this hypothesis includes the observation of a potential hormetic response in the nitrate salt exposure

BrdU ELISA experiments performed in this study (see section 5.1.2.2), the low alternate salt concentrations required to evoke the effect, and the magnitude of the response compared to the control group. Calabrese (2005) noted that many hormetic responses generally involve an elevated response at approximately 150% of the control value; while not all of the responses observed in this experiment are of this magnitude, many of them are quite strong. The strength of the responses observed in this assay reduce the perception that these responses are due to biological variation, though the possibility still exists. More experimentation is required to determine if the observed results indicate a *bona fide* hormetic response. However, the results from the 48 hour HepG2 alternate salt BrdU ELISA assays appear to support the hypothesis that a hormetic response may exist.

Based on the results of this assay, the relative toxicity rankings of the alternate salts for effects on HepG2 cell proliferation after a 48 hour exposure are $NaNO_3$ < CH_3COOK < CH_3COONH_4. Unfortunately, 48 hour BrdU ELISA assays were not performed on HepG2 cells exposed to KNO_3 or NH_4NO_3, so direct comparisons between the nitrate salts and alternate salts experiments cannot be made. However, using the 24 hour and 72 hour KNO_3 and NH_4NO_3 exposure data, the following observations may be made. The EC_{50} value for $NaNO_3$ was not calculable based on the available data, but could be assumed to be by far the highest observed for any of the HepG2 BrdU ELISA assays. Both acetate salts tested in this assay produced EC_{50} values (CH_3COOK EC_{50} = 1248 mg/L, CH_3COONH_4 EC_{50} = 943 mg/L) that fell between the EC_{50} values observed for the 24 hour (EC_{50} = 1943 mg/L) and 72 hour (EC_{50} = 730 mg/L) KNO_3 HepG2 BrdU ELISA assays. The EC_{50} values produced by the 24 and 72 hour NH_4NO_3 HepG2 BrdU

ELISA assays, however, were nearly the lowest of all the HepG2 BrdU ELISA assays at approximately 734 mg/L and ~2.5 mg/L, respectively. Thus, it appears that the relative ranking of toxicity in substances used in HepG2 BrdU ELISA assays is $NaNO_3$ < KNO_3 ≈ CH_3COOK < CH_3COONH_4 < NH_4NO_3. However, this is not an exact ranking, as data from different timepoints were used to calculate this relationship.

In summary, the results of the 48 hour HepG2 alternate salt BrdU ELISA assay showed that CH_3COONH_4 was the most toxic to HepG2 cells, followed by CH_3COOK and $NaNO_3$. This may have been due to the toxic effects of the acetate anion. Surprisingly, potassium acetate was found to be more toxic to HepG2 cells in the Neutral Red viability assay, but ammonium acetate was found to have a greater detrimental effect on proliferation in this assay. The reasons for this finding are unknown. The two acetate salts produced similar steep, exponential declines in cell proliferation, while $NaNO_3$ produced a linear, somewhat irregular, gentle decrease. Both acetate salts reduced HepG2 cell proliferation to a very low level at the highest concentration, while $NaNO_3$ exposure had only a minor effect on cell proliferation. An initial increase in cell proliferation in all alternate salts tested may have been indicative of a hormetic response, especially given the strong initial increase in proliferation compared to the control group. This hypothesis, however, requires further study. Finally, based on the EC_{50} values obtained from the HepG2 nitrate and alternate salt BrdU ELISA assays, the relative ranking of toxicity for each salt is $NaNO_3$ < KNO_3 ≈ CH_3COOK < CH_3COONH_4 < NH_4NO_3.

5.2.5 48 hour HEK293 alternate salt exposure – BrdU ELISA

In this assay, as in the 48 hour HEK293 alternate salt Neutral Red assay (see section 5.2.3), the acetate salt evoked much greater toxicity to the HEK293 cells than the nitrate salt. This is likely due to the toxicity of the acetate anion itself, as described in section 5.2.2. The relatively low toxicity of sodium nitrate could be due to the presence of sodium ion, which may have modified the response of the kidney cells as described in section 5.2.3. Because sodium and potassium management are closely linked in the kidney, it is possible that the high concentrations of sodium ion present in this assay may have triggered the same homeostatic mechanisms that were hypothesized in section 5.1.1.2, thus increasing resistance to nitrate toxicity. However, this is only a hypothesis and requires further investigation. An examination of the EC_{50} values for the HEK293 nitrate salt BrdU ELISA experiments reveals that the EC_{50} value generated for CH_3COONH_4 in this experiment (907 mg/L) falls between the 24 hour HEK293 BrdU ELISA NH_4NO_3 EC_{50} of 1442 mg/L and the 72 hour HEK293 BrdU ELISA NH_4NO_3 EC_{50} of 471 mg/L. Comparatively, the $NaNO_3$ EC_{50} obtained in this experiment (5636 mg/L) was much larger than any of the EC_{50} values obtained in the nitrate salt BrdU ELISA experiments. The closest EC_{50} value is that of the 72 hour HEK293 BrdU ELISA KNO_3 assay, at 3631 mg/L. No comparisons could be made with a 48 hour nitrate salt timepoint, as this timepoint was not used in the experimental nitrate salt BrdU ELISA assays performed with HEK293 cells. Based on the results of this experiment, ammonium acetate appears to have a greater adverse effect on HEK293 cell proliferation than sodium nitrate in a 48 hour exposure. When the EC_{50} values from the HEK293 experimental nitrate salt BrdU ELISA assays are considered, the relative toxicity scale appears to be $NaNO_3 < KNO_3 < CH_3COONH_4 \approx NH_4NO_3$. However, because the EC_{50}

values are not all obtained from exposures at the same timepoint, this ranking should be used only as a general theoretical guide.

Like the 48 hour HepG2 alternate salt BrdU ELISA assay described in section 5.2.4, a possible hormetic effect was observed in the CH_3COONH_4 group in this assay. Unlike the HepG2 assay, however, this effect was observed only in the CH_3COONH_4 group and not in the $NaNO_3$ group. The reason for this difference is not known, as a hormetic-like effect was noted for the $NaNO_3$ group in the HepG2 assay referenced above. It is possible that some cell type-specific response is responsible for mediating the hormetic effect; the current literature indicates that HepG2 cells have been observed to respond hormetically to some toxicants, but no descriptions of hormesis in HEK293 cells are available. An unknown property of HepG2 cells may make this cell type more prone to this kind of effect. There may be some interaction between cell type and the nature of the toxicant present, which may explain why CH_3COONH_4 produced this response in both cell types in the BrdU ELISA assay, but $NaNO_3$ only produced a hormetic-like response in HepG2 cells. Again, it is possible that the effect observed in this assay is an artifact, but the nature and strength of response is compatible with the range observed in prior hormesis studies (Calabrese, 2005).

In summary, ammonium acetate appeared to have a much greater toxic effect on cell proliferation in HEK293 cells than sodium nitrate under these experimental conditions. This may have been due to the toxicity of the acetate anion. The relatively mild toxic effects of sodium nitrate may have been due to the activation of homeostatic mechanisms in these cells, but further investigation is required to confirm or disprove

this hypothesis. A hormetic-like effect was observed in the CH_3COONH_4 group, but not the $NaNO_3$ group, at low concentrations. This observation differs from those made in the 48 hour HepG2 alternate salts BrdU ELISA assay, where the hormetic-like effect was observed in all three salts tested. The reason for the difference between the HepG2 and HEK293 assays are unclear, but may indicate that the observed hormetic-like effect may have a cell-specific and cation/anion-specific component. More research is needed to fully investigate and elucidate this phenomenon. The relative toxicities observed for experimental and alternate salts in the HEK293 BrdU ELISA assays are $NaNO_3 < KNO_3 < CH_3COONH_4 \approx NH_4NO_3$.

5.2.6 24 hour ammonium nitrate resazurin assay – HepG2 and HEK293

Compared to the 24 hour NH_4NO_3 Neutral Red viability assays for HepG2 and HEK293 cells, the resazurin assay produced a different pattern of response for both cell types. As shown in Figure 4.2, the 24 hour Neutral Red NH_4NO_3 assays demonstrated a steady quadratic polynomial decline in both HepG2 and HEK293 cells with increasing concentrations of NH_4NO_3. This is quite different from the almost parabolic decline observed in both cell types in the resazurin assay (see Figure 4.21). The EC_{50} values for each assay are also widely different; for the 24 hour Neutral Red NH_4NO_3 assay, the EC_{50} values were 3010 mg/L for HepG2 cells and 1807 mg/L for HEK293 cells. However, for the resazurin assay, the EC_{50} values were 4789 mg/L for HepG2 cells and 3974 mg/L for HEK293 cells. The percentage of control group viability observed at 5000 mg/L NH_4NO_3 is also quite different between the two assays. In the Neutral Red assay, the final viability values are 9% of the control group for HepG2 cells and 24% of the control group for HEK293 cells. However, the resazurin assay resulted in HepG2

viability at 58% of the control group and HEK293 viability at 14% of the control group at 5000 mg/L NH_4NO_3.

One of the most probable explanations for the differences between the two methods is the difference in the mechanism used by each assay to measure viability. As described in section 5.1.1.2, the Neutral Red assay measures viability through the uptake and trapping of an acidotropic dye in living cells. However, the resazurin assay measures cell viability through a metabolic mechanism. *In vitro*, resazurin enters cells by passive diffusion in its oxidized form (Jondeau *et al*., 2006). Once in the cytosol, it may be reduced by metabolic enzymes in the cytosol, mitochondria, or microsomal fraction (Gonzalez & Tarloff, 2001). Resorufin, the reduction product of resazurin, emits fluorescence at 590nm when excited, and can thus be used as a marker of cell metabolic activity and viability. Because the two methods measure different aspects of cell viability, it would be expected that the results would differ somewhat. Another explanation may arise from consideration of the composition of the culture medium used in these assays. Phenol red is added to the culture medium as a pH indicator, but is known to reduce sensitivity in the resazurin assay (Sigma-Aldrich, 2007b). To counteract this reduction in sensitivity, the resazurin assay was evaluated using a fluorometric method, as opposed to the less sensitive colourimetric method. Still, the presence of phenol red may have affected the sensitivity of the observed results. It must also be remembered that the Neutral Red data were obtained from pooling the results of three independent experiments, while the resazurin data are based on three wells per treatment tested in a single 96-well plate. Thus, variability may have also played some role in the differences observed between the two assays.

The reason for the differences between HepG2 and HEK293 viability at the highest NH_4NO_3 concentrations in both assays is unclear. Logically, it would be expected that the cell type most affected at the highest NH_4NO_3 concentrations in the Neutral Red assay would be the same cell type shown to be most affected in the resazurin assay; however, this is not the case here. In the 24 hour NH_4NO_3 Neutral Red assay, the HepG2 cells showed higher viability than the HEK293 cells except at the highest NH_4NO_3 concentrations, when the situation was reversed. In the resazurin assay, however, both cell types retained similar viability throughout the lowest NH_4NO_3 concentrations, but HEK293 viability was much lower than HepG2 viability at the highest NH_4NO_3 concentrations. The proportionate difference in viability between the two cell types at the highest NH_4NO_3 concentration is also very different between the two assays; a much larger difference exists in the resazurin assay compared to the Neutral Red assay. It is possible that exposure to NH_4NO_3 affected the metabolic activity of the HEK293 cells differently than that of the HepG2 cells, but more research is required to determine the actual reason behind this discrepancy.

Despite the observed differences, the general viability trend between the two assays is somewhat similar. Both assays show that HepG2 and HEK293 viability decrease as a function of increasing NH_4NO_3 concentration, and both assays demonstrate a slightly increased sensitivity of HEK293 cells for the majority of the concentrations tested. Each assay also demonstrates the similarity of response between the two cell types; even though the HEK293 cells appear to be more sensitive than the HepG2 cells, the general shape of the trendline for each cell type in each assay appears to be very similar. The onset of significantly decreased viability compared to the control group also appears to

be relatively close in both assays; in the Neutral Red assay, both cell types show significantly reduced viability at and above 3000 mg/L. In the resazurin assay, significantly reduced viability is noted at and above 3500 mg/L in both cell types. Although these points are not the same, they are similar. Based on these observations, it appears that although the two assays do not produce the same results in terms of EC_{50} values or graph trendline shape, they do indicate that a similar response is occurring.

Because two different viability assays indicate a decline in HepG2 and HEK293 cells exposed to NH_4NO_3, it is evident that exposure to NH_4NO_3 under these experimental conditions produces adverse effects in several processes required for cell viability. Although some differences exist between the results of the two assays, they are likely due to the different parameters measured in each assay, a possible confounding factor present in the cell culture medium, and the number of replicates performed for each assay. Insufficient evidence exists to explain the differences in relative toxicity between the HepG2 and HEK293 cells at the highest NH_4NO_3 concentrations, but this may be resolved with further research. The general trends revealed by each assay, however, are similar. Thus, both methods agree that increasing concentrations of NH_4NO_3 result in decreased viability in both HepG2 and HEK293 cells, and that the HEK293 cells appear more sensitive to these adverse effects than HepG2 cells in the majority of concentrations tested. The observation of the same general trend between the two different assays supports the reliability of the Neutral Red data obtained in these experiments, and indicates that nitrate may have effects on several different intracellular processes under these experimental conditions.

5.2.7 Summary of the alternate salt and media control assay results

To summarize the results of the media control and alternate salt assay results, it appears that the Neutral Red assay may be prone to some potentially statistically significant variability, especially in the 24 hour exposure timepoint. This effect appears to decrease with time, however, as no flasks were significantly different from all others tested in the 72 hour media control assay.

The HepG2 and HEK293 Neutral Red alternate salt experiments both demonstrated the increased toxicity of the acetate salts tested compared to sodium nitrate. While sodium nitrate produced relatively mild toxicity in both cell types compared to the acetate salts, comparisons of the KNO_3 and NH_4NO_3 Neutral Red exposure data showed the EC_{50} values to be close or comparable to those of the experimental nitrate assays. All Neutral Red alternate salt assays showed decreasing cell viability in both cell types with increasing alternate salt concentration. A comparison of the EC_{50} values obtained from the Neutral Red experiments with published rat oral LD_{50} values showed relatively good agreement, with the exception of sodium nitrate. The LD_{50} values for sodium nitrate are less than those of the other substances tested in these assays; however, the EC_{50} value for sodium nitrate is much higher than others obtained in the Neutral Red assays in this study. The reason for this discrepancy is unknown. A hormetic-like effect was observed in only the HepG2 alternate salt Neutral Red assay, but may be due to the different concentrations used in the HepG2 and HEK293 assays. Additional experiments are required with an expanded concentration range to determine if this effect is also present in the HEK293 alternate salt Neutral Red assay.

In the BrdU ELISA assays, the acetate salts also demonstrated increased toxicity to both HepG2 and HEK293 cells compared to sodium nitrate. Interestingly, while CH_3COOK showed greater toxicity in the HepG2 Neutral Red alternate salt assay, CH_3COONH_4 appeared to have a greater negative effect on cell proliferation in the HepG2 BrdU ELISA alternate salt assay. All alternate salts tested, however, produced a decrease in cell proliferation compared to control for all BrdU ELISA alternate salt assays. A hormetic-like effect was noted in both the HepG2 and HEK293 BrdU ELISA alternate salt assays, though it was not always present in the same salts tested. Both the HepG2 and HEK293 BrdU ELISA assays showed a similar relative toxicity ranking of the experimental nitrate and alternate salts used in the BrdU ELISA assays, based on the EC_{50} values obtained.

While some differences were observed between the results of the Neutral Red and resazurin NH_4NO_3 assays, the general nature of the response to nitrate was the same. Both cell types experienced decreases in cell viability compared to control with increasing concentrations of NH_4NO_3, and HEK293 cells appeared to be slightly more sensitive than HepG2 cells in both assays. Observed differences in response between the assays were likely due to the different parameters of viability measured by the two assays, the number of replicate experiments performed, and a possible confounding factor present in the cell culture medium. The accordance between the two viability assays serves to reinforce the reliability and accuracy of the Neutral Red assay, and indicates that ammonium nitrate may adversely affect several different intracellular processes under these experimental conditions.

5.3 Nitrate exposure and protein expression

Examination of the protein expression data shows little effect of nitrate on protein expression under these experimental conditions. Nitrate does not appear to cause significant changes in the expression of PCNA, Hsp70, Hsc70, or VEGF in either HepG2 or HEK293 cells. There does not appear to be an effect of nitrate salt composition on protein expression. Some minor differences in response, however, exist and deserve further investigation.

5.3.1 Response of liver cell line (HepG2) compared to kidney cell line (HEK293)

Generally, the HepG2 and HEK293 cell lines responded in a similar manner to nitrate exposure. Neither cell type showed overt changes in protein expression for any of the proteins tested, although slight variations were observed for one protein.

Both cell types showed a slight increase in expression of Hsp70 protein as nitrate concentration increased; however, this change was very subtle and little change was observed overall. Likewise, both cell types showed no consistent change in Hsc70 protein expression with increasing nitrate exposure. Examination of VEGF protein expression was conducted only in the HEK293 cell line, so no comparison between the cell lines can be made here. The expression of VEGF appeared to be very low in both cell types; attempts to visualize VEGF protein expression in HepG2 cells were not successful.

However, a slight difference in expression between the two cell types was noticed in the Western blots for PCNA protein. While the HepG2 cell line showed a slight increase in

protein expression with increasing nitrate concentration, the HEK293 showed a slight decrease in protein expression under the same circumstances. Although these changes were very subtle, they do represent a noticeable difference between the cell types. A potential reason for this difference could be the relative susceptibility of each cell type to nitrate toxicity. Because nitrate is absorbed from the small intestine, it is subject to first-pass metabolism. Thus, the liver may be exposed to high concentrations of nitrate and may have developed increase resistance to the toxic effects. The liver may also be exposed to absorbed nitrite, also, if nitrate is reduced *in vivo* and reabsorbed through the digestive system. However, because nitrite is very unstable, it may react with components of the gastrointestinal system or the blood before encountering the liver.

The kidney is also potentially susceptible to nitrate toxicity *in vivo*. Approximately 65-70% of nitrate is excreted unchanged in the urine (Bartholomew & Hill, 1984). This suggests that the kidney concentrates nitrate and may be exposed to higher concentrations than other organs or tissues. Urinary excretion is also an important route of elimination for nitrite (WHO, 1996), so it is possible that the kidney is also exposed to elevated concentrations of nitrite. It is also possible that a difference in the response of each tissue type to nitrate toxicity may explain the difference in PCNA expression; this is discussed further in section 5.3.3.

5.3.2 Effects of nitrate salt on protein expression

Based on the Western blotting results, there does not appear to be a significant difference in response to each nitrate salt (potassium or ammonium nitrate) within each cell type. Protein expression response after exposure to potassium nitrate was similar to

the response after ammonium nitrate exposure. It appears that, at these concentrations, the salt of nitrate used does not appear to significantly affect expression of the examined proteins. In the cell viability and proliferation assays (see sections 4.1 and 5.1), ammonium nitrate was more cytotoxic to both cell types compared to the same concentrations of potassium nitrate. However, such a response was not observed here. A possible explanation could be that the acute toxicity of the cation in the nitrate salt could overshadow the toxicity of nitrate at high doses, such as those used in the cytotoxicity studies. However, these protein expression assays used much lower nitrate salt concentrations, so the toxicity of the salt component may not have been evident. At these concentrations, and under these conditions, the choice of nitrate salt does not appear to significantly influence expression of the examined proteins. Further experiments with different nitrate salts may be helpful to determine if this observation may be generalized.

5.3.3 Nitrate exposure and Proliferating Cell Nuclear Antigen (PCNA) expression

In these experiments, a subtle increase in PCNA expression was observed with increasing concentrations of potassium and ammonium nitrate in HepG2 cells, while a subtle decrease in PCNA expression was observed in HEK293 cells under the same conditions. This finding may be significant for several reasons due to the varied and important roles that PCNA assumes in human cells.

Because PCNA is involved in replication, DNA repair, and cell cycle control, changes in protein expression can imply that different intracellular processes are occurring. An increase in PCNA could indicate cell replication, DNA repair, progression through the

cell cycle, or activation of pro-survival mechanisms. Conversely, decreased PCNA expression could indicate entry into apoptosis or a lack of proliferation. The expression changes observed in this study may be complicated by the 20 hour half-life of PCNA protein (Quiñones-Hinojosa *et al*., 2005) and the subtlety of the observed changes. It is possible that the results observed are due to biological or experimental variation, but because each result was obtained twice, and gels and blots were stained to ensure equal protein loading, this appears less likely.

Consideration of the cytotoxicity assay results when interpreting protein expression data may help clarify the responsible intracellular processes. For example, the increase in HepG2 PCNA could be due to an increased rate of proliferation in these cells; results from the BrdU proliferation assay (see section 4.1.2) do show increased proliferation (the 'hormetic-like effect') at some of these concentrations. Thus, it is possible that PCNA is elevated due to increased proliferation of HepG2 cells. Likewise, it may be possible that these HepG2 cells are progressing through the cell cycle, but more research is required to investigate this possibility given the negative effects of nitrate on cell viability and proliferation.. However, it may be plausible to infer that the elevated PCNA levels in HepG2 cells are due to the activation of DNA repair mechanisms. This conclusion appears to be supported by the observations of the cytotoxicity assays, and is logical considering the proposed mechanisms of nitrate toxicity. Several studies (see section 2.8) have implicated free radicals or reactive oxygen species, which may form adducts with DNA, in nitrate toxicity. As discussed in section 5.1, the possibility of a hormetic effect of nitrate in these experiments should be explored in future studies because it may explain why an increase in PCNA was observed in this system.

The decrease in PCNA expression in HEK293 cells may also be explained by considering the results of the cytotoxicity assays. Reduced PCNA expression is potentially indicative of a decrease in proliferation; however, *increased* proliferation was observed at these concentrations in some of the HEK293 BrdU ELISA assays. This is a puzzling finding which cannot currently be explained by this data; more research is needed to determine the reason for this discrepancy. Reduced PCNA expression may also indicate increased cell mortality, but this does not agree with the increase in viability seen in the KNO_3 Neutral Red cytotoxicity assays. It may be possible that the high concentrations of nitrate used in the cytotoxicity experiments created a false increase in viability by increasing the permeability of the cells to the Neutral Red dye, but more evidence is needed before this can be confirmed. The decrease in PCNA as a consequence of cell death is supported by the results of the NH_4NO_3 Neutral Red assays, which show a decrease in cell viability with increasing NH_4NO_3 concentrations. The expression of PCNA is also reduced before the initiation of apoptotic cell death, which is possible in this case. The results of the viability assays do not support this inference, so further experimentation would be required to determine if apoptosis was occurring. Examining the expression of caspase-3 or using a commercial apoptosis detection kit would provide more information on the apoptotic status of the cells.

Several potential explanations for the discrepancy in PCNA expression between HepG2 and HEK293 cells exist. It is possible that HEK293 cells are more sensitive to nitrate toxicity than HepG2 cells, and thus enter the cell death pathway sooner. This, however, assumes that the decrease in PCNA in the HEK293 cells is indicative of apoptotic cell death. The HepG2 cells may be more resistant to apoptotic cell death because they are a

tumour cell line, which generally demonstrate increased resistivity. Likewise, many tumour-derived cell lines show PCNA overexpression (Paunesku *et al*., 2001), so it is possible that small changes in PCNA expression were masked in the HepG2 cells.

Generally, the changes in PCNA expression are very subtle and probably do not have great biological significance. However, the relationship between increased PCNA expression, increased proliferation observed in the BrdU ELISA assay, and a potential hormetic effect in HepG2 cells holds interesting potential and should be explored further. Although PCNA is involved in many intracellular processes, the small magnitude of the changes does not suggest a change in cellular function as a result of nitrate expression under the experimental conditions used in these assays. The difference between PCNA expression at nitrate concentrations below the MAC, and those above the MAC appear negligible; the overall effect is a trend, not a dramatic difference between individual treatments. Thus, these experiments do not support the hypothesis that significant changes in PCNA expression occur above and below the current MAC for nitrate in drinking water. The differing expression patterns in the cell lines used are interesting, and may indicate different intracellular responses to nitrate exposure or differing susceptibility to nitrate toxicity.

5.3.4 Nitrate exposure and Inducible Heat Shock Protein 70 (Hsp70) expression

In these experiments, a very slight increase in Hsp70 expression was observed with increasing concentrations of potassium and ammonium nitrate in both HepG2 and HEK293 cells.

An increase in Hsp70 expression is normally associated with a "classical" stress response; the cell undergoing stress has mobilized chaperone proteins to protect cellular proteins and polypeptides from denaturation. In this case, increased Hsp70 expression could indicate that the cells are responding to nitrate-induced damage, or that anti-apoptotic mechanisms have been activated. However, such a definite response is not observed in these experiments; the elevation of Hsp70 protein levels is subtle, if it is even indeed significant.

From the cytotoxicity assays, it is evident that nitrate is toxic to both cell lines, so the absence of a significant increase in Hsp70 is puzzling. One potential explanation is that the concentrations of nitrate used in the assays were not sufficient to induce significant toxicity; while it appears from the cytotoxicity studies that significant decreases in viability and proliferation are not observed at these concentrations, some decreases are still observed for most of the exposures. Other authors (Salminen *et al*., 1996) have found that HepG2 cells do not always show an increase in Hsp70 when exposed to hepatotoxic compounds. The same authors demonstrated that HepG2 cells are *capable* of Hsp70 induction after toxic insult, but that it does not always occur with all toxicant exposures. It is possible that nitrate acts upon these cells in a manner which does not induce Hsp70 production. It is also important to remember that Hsp70 is often overexpressed in cancer cell lines (Parcellier *et al*., 2003; Jäättelä, 1999), so any response may have been masked in the HepG2 samples. This finding explains why Hsp70 is visualized in the HepG2 control group; other authors (Schueller *et al*., 2001) have also found high baseline Hsp70 expression in HepG2 cells.

An interesting mechanism of Hsp70 induction has been proposed by Salminen *et al*. (1996). These authors suggest that ROS or reactive metabolite adduction to cellular proteins induces the production of Hsp70 protein. Nitrate is thought to exert toxicity through a ROS-mediated mechanism, so a Hsp70 mediated response would be expected in these experiments. A possible explanation might be that nitrate is not converted to nitrite in culture; this is a crucial step in the formation of ROS arising from nitrate. *In vivo*, reduction to nitrite occurs via reduction by oral or gut flora, but none of these are likely present in cell culture. Antibiotics were added to the growth medium in our experiments to prevent microbial growth, so it is unlikely that reducing bacteria are present. Bacterial contamination of media is visible as a white fluffy precipitate, so it is unlikely that it would go unnoticed. A small amount of nitrate reduction could be occurring *in vitro* through cellular processes, which could potentially produce a small amount of ROS-induced damage and explain the small increases in Hsp70 observed. It is obvious that nitrate is capable of inducing toxicity in both cell types, but perhaps not at the concentrations used in the protein expression experiments.

Under these experimental conditions, exposure to potassium and ammonium nitrate does not appear to invoke a classical induced heat shock protein-mediated stress response. Whether this observation is due to a toxic mechanism which does not involve Hsp70, or that nitrate is not converted into nitrite *in vitro* and thus cannot invoke Hsp70 expression is unclear. It is also possible that a very subtle heat shock response is occurring due to a small amount of oxidative stress caused by limited reduction of nitrate to nitrate. Further experiments which characterize the specific molecular mechanism of nitrate toxicity *in vitro* in this experimental setup are required.

5.3.5 Nitrate exposure and Heat Shock Cognate Protein 70 (Hsc70) expression

After exposure to potassium and ammonium nitrate, neither HepG2 nor HEK293 cells demonstrated significant changes to Hsc70 expression in these experiments. Some slight variation was observed, but no definite patterns were discernible.

Like Hsp70, Hsc70 is a member of the heat shock protein 70 family; however, Hsc70 is constitutively expressed, and retains some distinct functions (see section 2.9.3). Observing Hsc70 expression permits examination of the baseline status of Hsp70 proteins within the cell, which may be compared to the expression of inducible Hsp70. In this way, a distinction between the activity of inducible and constitutive heat shock proteins can be made, allowing a greater understanding of intracellular processes occurring as a result of nitrate exposure.

Generally, no change in Hsc70 expression was observed in either cell type, over all exposures of both potassium and ammonium nitrate. Although the strong Hsp70 signal observed in the control (0 mg/L NO_3^-) groups was somewhat puzzling, the strong Hsc70 signal in these groups is logical because Hsc70 is a constitutively expressed protein. Like Hsp70, Hsc70 is also strongly overexpressed in many cancer cell lines (Hfaiedh *et al*., 2005), which may mask small changes in protein expression. Considering the results of both the Hsp70 and Hsc70 protein expression assays, it appears that the Hsc70 expression does not change appreciably with exposure to nitrate, while Hsp70 protein levels may experience a slight elevation with increasing nitrate concentrations. This suggests that there may indeed be a slight induced heat shock response in cells exposed to nitrate under these experimental conditions. Because the experimental protocol was

able to detect the consistent expression of the constitutive protein (Hsc70), changes in the expression of the inducible protein (Hsp70) likely indicate an actual change due to a stress response rather than experimental variation.

Under these experimental conditions, exposure to nitrate does not appear to affect the expression of Hsc70 in HepG2 and HEK293 cells. This observation is expected since Hsc70 is a constitutively expressed protein, and levels are not anticipated to change significantly between treatment groups. Establishing baseline Hsc70 expression allows comparison to Hsp70 levels, and allows comparison between the constitutive and inducible members of the Hsp70 family. Because the Hsc70 expression remained constant while the Hsp70 expression increased slightly, it is likely that a small stress response occurred in the cells exposed to nitrate under these experimental conditions.

5.3.6 Nitrate exposure and Vascular Endothelial Growth Factor (VEGF) expression

Due to difficulties in visualizing the VEGF protein, only expression data from the HEK293 cell line was obtained. No significant changes in protein expression as a function of nitrate exposure were observed in either the potassium or ammonium nitrate treated groups; however, the ammonium nitrate blot was extremely faint and only visible when the film was placed on a lightbox. Visualization of the VEGF protein was very difficult; a high concentration of primary antibody (1:200) was required, and each blot was exposed to film overnight. Despite these measures, most blots did not produce usable films, and only one film was obtainable for each assay. Due to these difficulties, any conclusions based on this data must be interpreted with caution.

While considering the limitations of the data, several observations may be made. Nitrate does not appear to have a direct role in the induction of angiogenesis; however, studies of patients with lung (Colakogullari *et al*., 2006) and breast (Coşkun *et al*., 2003) cancer show correlations between serum VEGF levels, nitrate/nitrite concentrations and angiogenic activity. Nitrate and nitrite are measured in these studies to serve as an indicator of nitric oxide levels, so it appears that nitrate is not directly involved in the angiogenic process or in the induction of VEGF expression. However, because nitrate and nitrite may serve as a source of nitric oxide (see section 2.8), the possibility of indirect effects must still be considered. Increased VEGF expression may also increase local concentrations of nitrate and nitrite via increased nitric oxide synthase activity, which produces nitric oxide (Voelkel *et al*., 2006). Nitrate and nitrite are both products of nitric oxide breakdown. In these experiments, however, nitrate exposure did not appear to induce VEGF expression. Likewise, it does not appear that nitrate exposure induced a hypoxic response in the treated cells, since hypoxia induces VEGF expression (Ribatti, 2004).

It is not clear why the expression of VEGF protein was so low in these experiments. *In vivo*, VEGF plays important roles in both fetal and adult human kidneys, including maintenance of cells and microvasculature (Kim & Goligorsky, 2003). In the liver, VEGF plays an important role in restoring function after liver damage or partial hepatectomy (van den Heuvel *et al*., 2006). In many human cancers or cancer cell lines, VEGF is highly overexpressed (Ribatti, 2004); however, this was not seen with the HepG2 and HEK293 cells used in these experiments. Because increased VEGF expression is indicative of a pro-survival cascade (Mercurio *et al*., 2005), it does not

appear that the cells in these experiments have activated such a cascade. The nitrate concentrations used in the protein expression assays do not appear to evoke significant cytotoxicity in the viability or proliferation assays; however, some toxicity still occurs, so the absence of a pro-survival VEGF cascade is somewhat puzzling. It may be possible that toxicity resulting from nitrate exposure may not involve a VEGF-mediated pathway. Likewise, the proposed relationship between nitrate exposure and cancer development may not function on a VEGF pro-angiogenic pathway, if such a relationship even exists.

5.3.7 Nitrate exposure and protein regulatory mechanisms

Another explanation for the observed changes in protein expression seen for PCNA and Hsp70 may involve alterations in transcriptional or translational regulation of these proteins. It is possible that nitrate or its metabolites may have changed or contributed to the protein expression changes observed here. For example, Mirvish (1995) speculates that exposure to N-nitroso compounds may induce mutations in p53, a major control of PCNA expression within the cell (Paunesku *et al*, 2001). Low to moderate p53 expression induces PCNA expression, but high p53 expression has the opposite effect (Paunesku *et al*., 2001). N-nitroso compounds have been speculated to be products of nitrate metabolism under specific conditions (Spiegelhalder *et al*., 1976; Vermeer & Van Maanen, 2001; see section 2.8.1 for more details), though the presence or amount of N-nitroso compounds in this *in vitro* system is unknown.

However, this hypothesis does not explain why the HepG2 and HEK293 cells showed differing changes in PCNA expression. It is possible that one cell type is more resistant to p53 damage than another, or that p53 expression is different between these two cell

types. More research must be conducted to determine whether nitrate induces a change in PCNA expression through interactions with the transcriptional or translational regulatory systems. It is also important to note that both post-transcriptional and post-translational regulation of PCNA also occurs (Paunesku *et al*., 2001), which may explain why the HepG2 PCNA protein expression in this study showed the same trend as the PCNA gene expression observed by Bharadwaj *et al*. (2005), but at a greatly reduced magnitude.

Interestingly, a study by Chin *et al*. (1997) found that nitric oxide significantly upregulates VEGF mRNA production in HepG2 cells. Nitric oxide breaks down to form nitrate and nitrite, and there is some evidence that nitrate may also serve as a source for nitric oxide production (McKnight *et al*., 1997; Iijima *et al*., 2002). Chin *et al*. (1997) found that VEGF mRNA production increased significantly after approximately 6 hours of nitric oxide exposure, with a maximum response occurring after 12-24 hours of exposure. Based on the close relationship between nitrate and nitric oxide, it is possible that nitrate may have a similar effect on HepG2 cells. This finding is interesting because no such increase was observed in the current experiment, and previous work by Bharadwaj *et al*. (2005) had shown downregulation of the VEGF gene in HepG2 cells after a 24 hour potassium nitrate exposure. The reason for an apparent lack of effect in the current study and an opposite effect observed by Bharadwaj *et al*. (2005) is unclear; perhaps nitrate does not have the same effect as nitric oxide in this system. Given the difficulty encountered in producing reliable Western blots of VEGF protein, it is also possible that the effect was inobservable under the current protocol. More research into

the presence or absence of nitric oxide in this experimental system, and the responses of VEGF mRNA and protein to nitrate exposure will provide more insight into this effect.

Protein-protein interactions may have also influenced the expression changes observed in these experiments. The INhibitor of Growth, or ING family of proteins, has been shown to greatly influence cell growth and proliferation, apoptosis, and ageing (Russell *et al*., 2006). Interestingly, ING proteins have been shown to interact with both PCNA and Hsp70 *in vitro*. Upon cell damage, ING proteins bind to PCNA, which is thought to switch its function from DNA replication to DNA repair. ING proteins have also been shown to interact with p53, a major regulator of PCNA, although it is unclear how this interaction would affect PCNA protein expression *in vitro*. The actions of ING proteins are thought to be cell-specific, which may explain why PCNA expression increased in HepG2 cells but decreased in HEK293 cells. The action of ING proteins on chromatin compaction has been shown to induce Hsp70 expression (Feng *et al*., 2006), which was observed in the current set of experiments. The presence of ING proteins in HepG2 cells has been shown by Zhang *et al*. (2004), but no literature is currently available for the presence of ING proteins in HEK293 cells. However, because ING proteins are highly conserved and present in many different species (Russell *et al*., 2006), it is likely that they are present in the HEK293 cell line. Based on this information, it is possible that ING proteins may have been involved in the protein expression changes which resulted from nitrate exposure in these experiments. However, nitrate has not been shown to directly interact with ING proteins; this may be due to the relatively recent discovery of ING proteins and a lack of current information rather than the absence of a relationship.

Further study into the role of ING proteins and nitrate-mediated changes in protein expression in human cell lines is required to determine the relationship between the two.

In summary, it appears there is some evidence that nitrate metabolites could potentially affect the transcriptional or translational mechanisms of the proteins examined here. Based on the pattern of effects, it is also possible that protein-protein interactions may have played a role. However, the current experimental design did not provide any information about the presence of nitrate metabolites in this *in vitro* system, so specific details are unknown. Identification and quantification of nitrate metabolites in this system is vital to understand how these might affect protein expression, and such an investigation should be performed in future studies of this subject.

5.3.8 Effect of exposure time on protein expression changes

Although these experiments have provided interesting insight into the effects of nitrate exposure on protein expression in human cell lines, they should be regarded as a preliminary investigation only. Because only a 24 hour nitrate exposure period was used, all nitrate-induced changes in protein expression may not have been observed, especially in proteins with long half-lives. For example, the half-life of the PCNA protein is 20 hours (Quiñones-Hinojosa *et al*., 2005). The full extent of PCNA expression change may not have been visible after only a 24 hour period, as protein present at baseline might still remain in the cell.

The induction timeframe of Hsp70 protein appears to differ based on the degree of stress; a study by Lovell *et al*. (2006) showed an almost-immediate increase in Hsp70

expression in human leukocytes exposed to 40°C conditions, but induction took several hours at 37°C. Thus, if Hsp70 expression time is dependent upon the degree of stress present in the cell, it may take longer for cells treated with low concentrations of nitrate to fully express Hsp70 compared to cells treated with higher nitrate concentrations. Because a short exposure time was used in these experiments, it is possible that not all Hsp70 induction was observed. The expression of Hsc70 protein was not expected to change as it is constitutively expressed; thus, it is unlikely that the short exposure time negatively affected the Hsc70 assays.

However, a 24 hour period may have been sufficient to visualize changes in VEGF protein expression; a study by Baek *et al*. (2000) demonstrated an increase in VEGF protein after HepG2 cells were exposed to hypoxic conditions for 24 hours. However, VEGF mRNA levels increased after up to 36 hours of hypoxia, so the full expression change may not have been captured in a 24 hour window. It is unknown whether nitrate is as effective in provoking changes in VEGF expression as hypoxia; if not, additional exposure time may be needed to fully measure changes in expression in this assay. While it is not definitively known whether a 24 hour exposure period was sufficient to examine all protein expression changes in this assay, the addition of longer exposure periods would be beneficial to measure any changes in protein expression.

Based on information available in the literature, it is possible that a 24 hour nitrate exposure was not sufficient to fully examine protein expression changes in this system. Due to long protein half-lives, lag time between cell stress and expression induction, or a sustained change in protein expression, it would be advisable to add both longer and

shorter exposure periods to this system to examine protein expression changes. This would also provide information about how protein expression changes with time. Although these studies provided valuable information about the effect of nitrate exposure on the expression of specific proteins, the addition of more timepoints would greatly increase understanding of this effect.

5.3.9 Summary of the effect of nitrate exposure on protein expression

Overall, it appears that nitrate exposure under the conditions used in these experiments does not appreciably affect the expression of PCNA, Hsp70, Hsc70, or VEGF. With the exception of a slight difference in PCNA expression, the HepG2 and HEK293 cells responded in a similar manner. No significant indications of tissue-specific responses for the proteins examined were observed. Likewise, the choice of nitrate salt did not appear to significantly affect protein expression. No appreciable change in protein expression as a function of nitrate exposure was observed for Hsc70 and VEGF. A slight elevation in PCNA expression was observed in HepG2 cells exposed to nitrate, while a slight decrease was observed in HEK293 cells under the same conditions. Explanations for this observation may include differing susceptibility or response to nitrate exposure, but the changes are likely not biologically significant. A slight increase in Hsp70 expression was observed for both cell types, and may be indicative of a small stress response. It is possible that nitrate or its metabolites affected the transcriptional or translational regulatory mechanisms of these proteins, although more research is needed on this topic. Additional exposure timepoints may also be necessary in order to observe all changes arising from nitrate exposure. It does not appear that the expression of these proteins is affected at nitrate concentrations below the regulatory limit for nitrate in drinking water.

While these results are promising in terms of water safety and human health, more research is required to fully determine the effect of nitrate exposure on human protein expression.

5.4 Relation of results to current knowledge

Relating the current studies to knowledge present in the literature is challenging, as few recent studies have been performed to examine the role of nitrate on human health. Studies which examine the molecular and/or cellular effects of nitrate exposure on human cells are practically non-existent. However, several comparisons may still be made with studies closely related to the current work.

The original basis for the current study was the work performed by Bharadwaj *et al*. (2005). That paper examined HepG2 cells exposed to potassium nitrate for 24 hours; no studies with ammonium nitrate, HEK293 cells, or longer exposure periods were performed. Also, the Bharadwaj *et al*. (2005) study used nitrate concentrations between 3-1000 mg/L KNO_3, as opposed to the 1 μg/L-5000 mg/L nitrate concentrations used in this study. Several similarities were found between the current work and the findings of Bharadwaj *et al*. (2005). Through comparison of the common concentration range between the two data sets, it appears that the responses of HepG2 cells to a 24 hour potassium nitrate exposure were similar in both studies.

In the Neutral Red viability assay, both data sets showed a gradual increase in viability between the control group and the 1000 mg/L KNO_3 group, and a viability slightly greater than the control group at the 1000 mg/L KNO_3 exposure level. Both graphs

showed a similar trend in viability compared to the control group and lack of data points significantly different from the control group over the concentration range. Some differences, however, were also apparent. The different concentration ranges used in the two studies did present difficulties for data interpretation; while the Bharadwaj *et al*. (2005) work used 6 concentration values between the control group and the 1000 mg/L KNO_3 group, the current work used only the control group, 500 mg/L, and 1000 mg/L KNO_3 concentrations within that range. Thus, determination of similarities or differences between the data sets was slightly more difficult. The previous work found a sharp increase in viability at the 150 mg/L KNO_3 concentration, but because a similar concentration was not tested in the current study, a comparison cannot be made. Despite these differences, the overall trend in cell viability is similar between the two works.

The comparison between the BrdU ELISA assays in the current study and the previous work by Bharadwaj *et al*. (2005) was slightly easier due to the better overlap of tested KNO_3 concentrations. In the previous work, 6 concentrations between 0 mg/L and 1000 mg/L KNO_3 were used; in the current work, 8 concentrations were tested in the same range. Both data sets showed a gradual decline in proliferation compared to control over the entire concentration range, and the proliferation value at 1000 mg/L KNO_3 exposure was similar for the two groups (between 60%-72%). However, there were also obvious differences between the two data sets which require exploration. No initial spike or increase in proliferation was seen in the previous work, compared to the possible hormetic effect observed in the current study. Elevated proliferation values compared to control were observed at 3 mg/L, 45 mg/L, and 250 mg/L KNO_3 concentrations in the current work; however, these concentrations evoked proliferation values lower than the

control value in the previous work, with values at about 60% of the control group proliferation. It is possible that these differences arose because the Bharadwaj *et al*. (2005) data was the result of pooled values from three independent replicate experiments, and the current work was the result of one representative experiment from a group of three independent replicate experiments. As discussed in section 5.1.2.1, the initial increase in proliferation/proposed hormetic response was not observed in all of the replicate experiments for each combination of cell type and nitrate salt. It is possible that the proposed hormetic response did not occur in the experiments performed by Bharadwaj *et al*. (2005), or that it was an artifact in the current study.

Also, the Bharadwaj *et al*. (2005) study found significantly different proliferation compared to the control group in each nitrate concentration tested, while the current study did not find any significantly different data points in the same concentration range. The proliferation values compared to control in the previous work are all depressed, while the proliferation values in the current study fluctuate both above and below the control value throughout the same concentration range. This may be due to the differences in the cell cultures selected for each body of work; while the Bharadwaj *et al*. (2005) study used only cell cultures subjected to the same number of passages, the current work used cells of different passage numbers but attempted to use cells of similar age when possible. While this may have introduced additional variability and error into the current work, all cell cultures used in the current study were within the 10-passage limit proposed by Wilkening & Bader (2003) to limit passage-related variability effects. Again, the use of pooled data in the previous work and representative data in the current work may also have contributed to the discrepancy.

After consideration of all the similarities and differences between the previous work by Bharadwaj *et al*. (2005) and the current work, the two sources appear to be in reasonable agreement regarding the cytotoxic effects of a 24 hour KNO_3 exposure on HepG2 cells. Although comparison was hampered somewhat because of the different KNO_3 concentration ranges chosen in each assay, the Neutral Red data appeared to be in the greatest agreement between the two data sets. While the BrdU ELISA data showed less agreement between the two groups, the general trend over the concentration ranges tested appeared similar.

Some comparisons may also be made between the protein expression assays in the current work and the gene expression assays in the previous research by Bharadwaj *et al*. (2005). In the previous work, cDNA microarray assays were used to determine changes in gene expression induced by a 24 hour KNO_3 exposure in HepG2 cells. Two genes examined in that paper were examined at the protein level in the current work: PCNA and VEGF. In the findings of Bharadwaj *et al*. (2005), PCNA expression increased significantly with increasing concentrations of nitrate in a 24 hour KNO_3 exposure. Conversely, VEGF expression decreased significantly with increasing concentrations of nitrate in a 24 hour KNO_3 exposure. In the current study, however, PCNA protein expression experienced only a slight increase in HepG2 cells with increasing KNO_3 concentrations over the same range as the previous study. The expression of VEGF in the current study could not be reliably determined because of difficulties visualizing the protein, so a comparison cannot be made. This finding is somewhat puzzling, considering the good correlation between the cytotoxicity data of the two studies. One potential explanation for the difference between the gene and protein studies is that a

difference in transcription does not necessarily involve a proportional change in translation. Thus, the gene expression could be elevated but little or no difference would be observed in the protein expression. This would explain why a large increase was observed in PCNA gene expression, but only a slight increase was observed at the level of the PCNA protein. It has also been shown that PCNA is subjected to post-transcriptional and post-translational regulation (Paunesku *et al*., 2001), which may have prevented the increase in gene expression from becoming an increase in protein expression. Despite the difference in magnitude, both studies showed an increase in PCNA expression in HepG2 cells exposed to increasing concentrations of KNO_3 in a 24 hour exposure. This indicates good correlation between both studies in terms of cytotoxicity data as well as gene/protein expression data.

A more recent study by Jondeau *et al*. (2006) explored the effects of several different water contaminants, including sodium nitrate, on RNA synthesis, cell viability, and ATP concentration in HepG2 cells after a 20 hour exposure to each toxicant. Cell viability was measured using the alamar blue assay, also known as the resazurin assay, which was also used in the current work. A detailed description of the work by Jondeau *et al*. (2006) may be found in section 2.10. Although the current study also used the resazurin/alamar blue assay, no examination of the effect of sodium nitrate on HepG2 function using resazurin was performed. However, an investigation into the effect of sodium nitrate on HepG2 and HEK293 viability was performed using the Neutral Red assay (see Figures 4.17 and 4.18, and sections 4.2.2.1 and 4.2.2.2) using a 48 hour exposure time. The EC_{50} value for a reduction in cell viability in the current work was 4429 mg/L for HepG2 cells; the EC50 value for 48 hour HEK293 $NaNO_3$ assay was not

reliably calculable based on the data available, but was 18557 mg/L when calculated from the best-fit trendline equation. Jondeau *et al*. (2006) found an IC_{50} value of 13599 mg/L for a 20 hour $NaNO_3$ exposure in HepG2 cells.

Because of the differing concentrations and timepoints used, a direct comparison between the two data sets is not possible; however, more general observations may be made. The Neutral Red assay for HepG2 cells exposed to sodium nitrate for 48 hours produced a sigmoidal viability curve, like that of Jondeau *et al*. (2006), as seen in Figure 4.17. The IC_{50} value from the Jondeau *et al*. (2006) paper and the EC_{50} value from the current work are quite different, although it is important to recognize that the sodium nitrate exposures were for substantially different amounts of time (20 and 48 hours, respectively). Despite the differences in exposure time, the EC_{50} value from the current work and the IC_{50} value from the Jondeau *et al*. (2006) paper both appear logical if several factors are taken into consideration. The resazurin and Neutral Red assays determine cell viability by different mechanisms; the resazurin assay measures metabolic activity while the Neutral Red assay measures dye uptake by living cells. The EC_{50} values for the 24 hour NH_4NO_3 Neutral Red and resazurin assays performed in the current work are quite different; the Neutral Red assay produced an EC_{50} of 1686 mg/L, while the resazurin assay produced an EC_{50} of 4789 mg/L. As discussed in section 5.4, such a difference may be expected due to the different mechanism of each assay. Logically, a difference might also be expected between the Neutral Red assay values in the current work and the resazurin assay values in Jondeau *et al*. (2006). When the difference in exposure time is also considered, the EC_{50}/IC_{50} values from the current work and Jondeau *et al*. (2006) appear reasonable. However, further experimentation

with both the Neutral Red and resazurin assays would need to be performed in order to fully determine the similarities and differences between the two data sets.

The potential for a link between nitrate exposure and chronic health effects, including cancer, diabetes, thyroid disorders, and teratogenic or reproductive effects, has been a hotly debated subject in epidemiology for several decades (see section 2.5). The results of these experiments *per se* do not provide definitive evidence to support or refute such a link. From the evidence collected in this study, it does not appear that nitrate concentrations at levels commonly found in Canadian or American municipal water supplies are capable of inducing cytotoxicity or significant changes in protein expression under the experimental conditions used here. However, many epidemiological studies examine populations which rely on private water supplies or wells for their drinking water. These private supplies may be contaminated with nitrate concentrations many times greater than the regulatory limit. Other epidemiological studies examine populations which inhabit severely nitrate-contaminated areas. Applying the results of this study become more difficult in these situations. From the values obtained for the cytotoxicity assays performed in this study, it is possible to conclude that nitrate concentrations equivalent to those found in some of the epidemiological studies are capable of evoking changes in cell viability and proliferation in HepG2 and HEK293 cells under the experimental conditions reported here. However, it is also extremely important to recognize the limitations of extrapolating *in vitro* data to *in vivo* situations. In these studies, there was no interaction with other tissues or body systems, presumably no bacterial conversion of nitrate to nitrite, only short exposure periods were used (compared to a person who might drink contaminated water for months, years, or

decades), and the cell lines which were used do not necessarily reflect the behaviour of human cells *in vivo*. Thus, it is not possible to conclude from this data that the nitrate concentrations which evoked cellular or molecular changes in these experiments are capable or not capable of producing the stated adverse health effects in humans. Larger, better-designed and controlled epidemiological studies are required before conclusions can be made about a potential link between drinking water nitrate exposure and adverse chronic health effects.

Because an increase in cell viability (as measured by the Neutral Red assay) was observed in HEK293 cells with increasing concentrations of nitrate in a short-term exposure, a discussion of the proposed beneficial effects of nitrate exposure is required. Several researchers have proposed that nitrate exposure is beneficial due to its bactericidal effects in the gastrointestinal tract or potential vasodilatory action from its conversion to nitric oxide (McKnight *et al*., 1999). Others have proposed that nitrate exposure does not pose a risk to human health, and the regulatory limits for nitrate in drinking water should be raised, not reduced (Avery, 2001; Avery & L'hirondel, 2003; L'hirondel *et al*., 2006). These results could be interpreted to support these viewpoints; however, the context of the results in the current survey must be carefully considered before conclusions about any beneficial effects of nitrate may be made. As discussed in section 5.1.1, the reason for the apparent increase in viability in HEK293 cells is not immediately apparent. Proposed hypotheses include the presence of an artifact or physiologically-induced changes in cell membrane permeability due to the high potassium concentration present in those exposures. When the BrdU proliferation data is also considered, the situation becomes more complicated; HEK293 cells show a

decrease in cell proliferation at some concentrations below the current regulatory limit but an increase at others. Examination of the protein expression data reveals a slight decrease in PCNA expression and a slight increase in Hsp70 expression in HEK293 cell cultures under the same conditions. Taking all of this data into account, it does not appear to support the positions held by McKnight *et al*. (1999), Avery (2001), Avery & L'hirondel (2003), and L'hirondel *et al*. (2006). Because the other data indicates a possible adverse effect under these conditions, given the large body of literature which indicates that nitrates have the potential to be harmful at high concentrations, and given the presence of a potential alternative explanation for the apparent increase in viability, these results do not indicate that nitrate has had a beneficial effect on HEK293 cells under these conditions.

Although the current availability of information on the molecular and cellular effects of nitrate on human cells is limited, the current study appears to agree well with what is known. This work supports and extends the conclusions made by Bharadwaj *et al*. (2005), and complements research performed by Jondeau *et al*. (2006). However, this study is unable to clarify whether or not the presence of nitrates in drinking water increases the risk of cancer or other chronic health conditions in humans. Likewise, these results do not support the position of some researchers that drinking water nitrate has a beneficial or negligible effect on human health. It is hoped that the results obtained here have provided valuable information about the effects of nitrate on human cell lines, and will inspire future research so that all aspects of nitrate toxicity may be understood.

5.5 Implications of results

5.5.1 Human health

Based on the results of these assays, nitrate does not appear to pose a significant risk to human health (as measured in these experiments) at concentrations below the MAC/MCL values, or at concentrations commonly observed in Canadian public water supplies. Significant decreases in cell viability and proliferation were not observed in either HepG2 or HEK293 cells at nitrate concentrations that would be commonly observed in all but the most contaminated areas. The HepG2 72 hour ammonium nitrate BrdU ELISA proliferation study did appear to have an EC_{50} value below the MAC; however, this determination was made from one representative experiment and may have been subject to significant error (see section 5.1). The EC_{50} values for the remainder of the assays were concentrations several orders of magnitude above the nitrate concentrations normally observed in Canadian municipal drinking water. Acute toxic effects, such as methemoglobinemia, would likely occur at concentrations below those required for cytotoxic effects as observed in this study.

Nitrate concentrations above and below the current MAC did not have appreciable effects on the expression of VEGF, PCNA, Hsp 70, and Hsc70 in both HepG2 and HEK293 cells. The expression of these proteins did not appear to change significantly from the control to the highest exposure level. Expression of PCNA and Hsp70 did change subtly between exposure levels; however, whether this observation was due to a change in protein expression or simple variation is unknown. These results suggest that nitrate exposure, both above and below the current drinking water limits, does not

appear to significantly affect the expression of VEGF, PCNA, Hsp 70, and Hsc70 in the HepG2 and HEK293 human cell lines under these experimental conditions.

Caution must be exercised, however, in the extension of *in vitro* results to *in vivo* situations. The use of cell culture in experiments such as these is an acceptable practice in toxicological studies, and both HepG2 and HEK293 cells have been used extensively in toxicity testing. However, the use of cell culture lines removes the opportunity to examine interactions with other cell types, organs, or body systems. There may be significant interactions which occur *in vivo* which were not represented in an *in vitro* study.

The timescale of these experiments is also an important consideration when extrapolating these results to *in vivo* effects. A consumer of nitrate-contaminated water will be exposed to nitrate every day, potentially for decades or a lifetime. For practical purposes, however, it is not possible to culture HepG2 and HEK293 cells for extended periods of time. The longest exposure period used in this study was 72 hours; this permitted exposure of the cells without the need to passage the culture or renew the media during the exposure. A valuable addition to further studies would be a protocol which allows for longer exposure times in these cultures.

Nitrate, in particular, is thought to exert its toxic effects through *in vivo* conversion to nitrite, a substance with a much greater toxicity. This conversion normally occurs when bacteria in the digestive tract reduce nitrate to nitrite, which is then recirculated throughout the body. It is unlikely, however, that such bacteria are present in the cell

cultures used in this experiment, since antibiotic agents are added to the growth media to discourage bacterial growth. Whether nitrate is converted to nitrite in the cultures used in these experiments is not known, though it appears unlikely to occur in large amounts. Although this permits an examination of the effects of nitrate itself, it may not adequately reflect conditions found *in vivo*.

Based on these experiments, it appears that nitrate concentrations commonly found in Canadian municipal water supplies do not induce significant cytotoxicity or expression changes of PCNA, Hsp70, Hsc70, or VEGF in HepG2 and HEK293 cells under these experimental conditions. This may suggest that nitrate does not pose a significant risk to human health under these conditions; however, more research is required to support this conclusion. It is important to note that direct extrapolation of these results to *in vivo* situations is unwise, as the use of cell cultures, limited exposure times, and the potential lack of nitrite formation represent significant departures from real-world situations.

5.5.2 Regulatory limits

Because nitrate does not appear to pose a significant risk to human health based on this research, no changes to the drinking water regulatory limits for nitrate can be proposed. The current drinking water standards appear to be acceptable, and the toxic endpoint of infant methemoglobinemia, used to develop the limit, appears to be sufficiently sensitive.

However, from the epidemiological evidence presented in the literature review (Chapter 2), there is conflicting evidence whether nitrate is involved in the development of

adverse health effects such as cancer, diabetes, and thyroid conditions. Some studies have observed significant effects of nitrate on disease incidence at concentrations below current regulatory limits. Others have determined that no significant increase in adverse effects have occurred in populations exposed to nitrate concentrations far above the MAC. This disparity indicates the need for further study with a larger case-controlled population; many of the conflicting studies mention the need for further study. Several of the proteins examined in this thesis are involved in cancer development or progression, but due to the inconclusive findings and the difficulty of applying *in vitro* results to *in vivo* situations, no conclusions can be made regarding cancer risk and nitrate concentration from this data.

An important consideration when developing regulatory limits for drinking water is that many contaminants may be present, and may interact in unexpected ways. This is particularly significant for rural private water supplies, which may have inputs of pesticides, fertilizers, agrichemicals, and other contaminants. Private water supplies may not be subject to regular monitoring, so contaminants may be present in unknown concentrations for unknown lengths of time. Interactions between drinking water contaminants may occur, but most studies focus on a single contaminant. A possible scenario may then arise where several contaminants are at legislatively acceptable concentrations, but which interact and possess additive or synergistic effects. Due to the propensity of some contaminants to occur together in drinking water, it is possible to erroneously conclude that only one contaminant is responsible, when in reality the concurrent or combination of the contaminants is to blame. This scenario was presented in a paper by Van Leeuwen *et al*., (1999) where the association with drinking water

nitrate and stomach cancer was proposed to be due to the concurrent presence of atrazine and the interactions between the two contaminants, not the concentration of nitrate alone. Thus, it is important to consider not only the concentrations of individual contaminants in drinking water, but also of concurrent contaminants and potential interactions which may occur.

This study does not provide evidence which supports a change to the current regulatory limit for nitrate in drinking water. However, a proposed association with drinking water nitrate and chronic disease or cancer risk is a persistent topic in epidemiological literature. It is possible that existing studies have not been sensitive enough to detect what may be a legitimate increase in risk, or that the observed effects are actually due to another contaminant or cause. Unfortunately, these experiments were not designed to resolve this issue; further, more detailed epidemiological studies are required. Studies which examine the prevalence and concentration of nitrate and other concurrent contaminants would be helpful to determine the actual risk to human health from each.

5.5.3 Drinking water policy

Although these results suggest that the current levels of nitrate present in the majority of Canadian municipal drinking water supplies do not appear harmful to human health, caution must still be observed. Private wells may contain nitrate concentrations in gross excess of current regulatory limits, and may not be tested regularly. The maximum nitrate concentration observed in a Saskatchewan private well in a one-year period (957 mg/L, as reported in Thompson, 2001) exceeded several of the EC_{50} concentrations for reduction of proliferation in both HepG2 and HEK293 cells in these experiments.

Severely nitrate-polluted areas of the world may achieve nitrate concentrations in excess of the EC_{50} values determined for cell viability and proliferation in these experiments.

Despite the results of this study, responsible drinking water policy should attempt to keep nitrate concentrations as low as reasonably achievable, and provide frequent monitoring to ensure nitrate concentrations do not exceed regulatory limits. Excess nitrate inputs should be eliminated or minimized, and efforts should be made to protect drinking water sources from contamination. When necessary, technologies such as reverse osmosis or ion-exchange should be used to treat drinking water to reduce nitrate content, or alternate sources of drinking water should be used. Users of private drinking water sources should be educated about their responsibility for water quality testing, and the importance of regular sampling and analysis.

5.5.4 Implications for rural health

Residents of rural areas are particularly vulnerable to the effects of nitrate toxicity – and drinking water contaminants as a whole – due to increased reliance on private water supplies. Municipal water supplies are not always available to rural residents, especially those in remote areas. Those using private water supplies may not be aware that it is their responsibility to have the supply tested, or of the importance of regular monitoring. Rural residents and their water supplies are subjected to a large variety of potentially harmful contaminants, including pesticides, fertilizers, manure runoff, and agrichemicals. Unfortunately, the majority of nitrate-induced infant methemoglobinemia cases appear to occur in rural areas, in families reliant on private wells for drinking water (see section 2.5.1). In many cases, the parents were not aware of the nitrate

contamination, and did not test their well regularly. Further compounding the problem, residents of rural areas also have less access to adequate and timely health care, especially in cases of emergency. Specialized care is often not available, and rural residents often do not have a choice of hospital or health care provider. Because rural residents must travel further to reach medical services compared to urban residents, accessing medical care can represent a significant financial hardship (Kinsely, 2002). In terms of nitrate drinking water toxicity, rural residents are at a distinct disadvantage – nitrate contamination appears to be more frequent and severe in rural private water supplies, and access to health care is not readily available should adverse effects occur. Strategies to counteract this problem could include increased education about the importance of water testing, proper well maintenance and hygiene, and harmful effects of drinking water nitrate. Educating rural health care professionals about the signs and symptoms of infant methemoglobinemia, and increasing the access of rural residents to timely and adequate health care could also help prevent nitrate-induced adverse health effects in vulnerable rural populations.

5.6 Future directions

Although this research has provided additional information about nitrate toxicity in human cell lines, further assays may be performed. The effect of nitrate exposure on cell viability may be further investigated by repeating all viability assays using the resazurin method. This will permit verification of the results seen with the Neutral Red method, as the resazurin assay measures metabolic activity, not dye uptake. Likewise, another proliferation assay (e.g. tetrazolium salt/MTT) might be used to verify the results of the BrdU ELISA assay used in these experiments.

Because of the uncertainty of the relative concentrations of nitrate and nitrite in the treatment solutions or cultures, the addition of an assay capable of quantifying these components would be useful. The Greiss reagent, which permits quantitation of both nitrate and nitrite, may be used to determine if *in vitro* reduction of nitrate to nitrite is occurring. This would be helpful in determining if observed toxicity is due to nitrate or nitrite, which is important considering the complex toxicokinetics and toxicodynamics of these two substances in *in vivo* testing.

As discussed in the previous sections, a hormetic-like effect was observed in several of the cytotoxicity assays. Additional testing would be useful to determine if this effect is a true hormetic effect or simply an experimental artifact. Because the effect was observed primarily in the BrdU ELISA proliferation assays, a likely avenue of further research would be to repeat these assays using low, closely incremented concentrations of nitrate. However, because one Neutral Red assay appeared to demonstrate the same effect when low nitrate concentrations were tested, additional low-exposure viability assays may also prove fruitful. Additional assays to examine the nature of the hormetic-like effect in HEK293 cells would be particularly beneficial, since a hormetic effect in this cell type has not yet been described in the literature.

To provide some context to the cytotoxicity and protein expression findings, it would be useful to determine if the exposed cells were undergoing apoptotic cell death. This would permit a greater understanding of the protein expression changes in the treated groups, as some of the observed changes could potentially be due to the onset of apoptosis. Several options for determining apoptotic status are available, including

Western blotting for caspase-3 or other apoptotic proteins, radioactive and non-radioactive DNA fragmentation assays, or ELISA-based assays which may detect DNA or histones in apoptotic cells.

Another potential approach would be to expand the number of cell types and proteins used in these assays. This would permit a greater exploration of tissue specificity, as well as permit examination on a tissue-by-tissue basis of all suspected sites of nitrate exposure-induced cancer. Potential human cell lines include those arising from the gastrointestinal system, such as the stomach and intestines, as this is the first point of contact for ingested nitrate and many suspected cancer sites are located here (see section 2.5.2.2). Additional proteins to be examined could be obtained from the previous work by Bharadwaj *et al*. (2005), which generated a list of genes affected by nitrate at the transcriptional level. A greater understanding of the effect of nitrate exposure may also be obtained by examining gene expression changes at the level of mRNA, in order to determine if changes in expression occur at the level of transcription or translation. This may help to explain discrepancies between the patterns of expression found in Bharadwaj *et al*. (2005) and the current work.

Eventually, it may be desirable to develop experiments which directly examine the effects of nitrate-contaminated drinking water on cell cultures or experimental organisms. This would permit a more environmentally relevant set of data, but may also lead to additional confounding issues. Drinking water, particularly rural private well water, is often a complex mix of contaminants, and can vary widely within a small area. Careful thought would have to be given to the location and time of sampling, and the

samples would need to be thoroughly analyzed to determine the type and concentration of potential toxicants. Additional difficulties may arise from developing a protocol to expose cell cultures directly to drinking water samples, as issues of bacterial contamination may be difficult to avoid. *In vivo* testing is also a possibility, and several studies have already examined the effects of *in vivo* nitrate exposure in mice and rats. However, any future studies would have to be carefully planned, as there are significant toxicokinetic differences between rodents and humans. For example, rodent gastric pH is higher than that found in humans (5.0 compared to 1.5), which may affect the formation of nitrosamines (NRC, 1995). Also, unlike humans, rats do not secrete plasma nitrate into saliva (NRC, 1995); this may have significant repercussions for nitrate reduction and recirculation. Despite these pitfalls, additional cell culture and *in vivo* testing may provide valuable information about the mechanisms of nitrate toxicity in environmentally relevant situations.

Although this research has explored several facets of nitrate toxicity, there remains much to be discovered. Possible future directions include expanding cytotoxicity assays, quantifying nitrate and nitrite concentrations *in vitro*, and determining the apoptotic status of treated cells. Examining additional cell lines or proteins may provide more information about tissue specificity and help relate this research to the work of others. Eventually, *in vitro* and *in vivo* studies with actual drinking water samples may provide additional information about nitrate toxicity in environmentally relevant situations. A greater understanding of nitrate toxicity will allow for the protection of sensitive subpopulations and improve drinking water quality for all users.

5.7 Conclusions

Based on the results of these assays, the following conclusions may be made:

I. Exposure to potassium, ammonium, or sodium salts of nitrate does not appear to have significant adverse effects on HepG2 and HEK293 cell viability and proliferation at concentrations at or below the current Maximum Acceptable Concentration (MAC) set by Health Canada (10 mg/L as NO_3^-). However, nitrate concentrations which may exist in highly polluted water sources, such as contaminated private drinking water wells, have evoked significant reductions in cell viability and/or proliferation under these experimental conditions.

II. The HepG2 and HEK293 cell lines appear to respond to nitrate exposure in a similar manner for the majority of assays performed. However, HEK293 cells appear to be less sensitive to KNO_3 exposure than HepG2 cells in the Neutral Red assays, but slightly more sensitive to NH_4NO_3 in the 24 hour Neutral Red assay. When all results are considered, HepG2 cells may be slightly more sensitive to nitrate toxicity than HEK293 cells. The increased resistance observed in HEK293 cells exposed to KNO_3 may involve the activation of tissue-specific homeostatic mechanisms, but more experimentation is required to explore this hypothesis.

III. Compared to potassium nitrate, ammonium nitrate evoked greater toxicity in both HepG2 and HEK293 cells in the viability and proliferation assays. This may be due to the elevated pH of ammonium nitrate solutions, or toxicity caused by the ammonium cation itself.

IV. Generally, a decrease in cell viability and proliferation was observed when the nitrate exposure time increased from 24 to 48 to 72 hours. The EC_{50} values obtained for the viability and proliferation assays decreased as exposure time increased.

V. A sharp increase in proliferation at low nitrate concentrations (a “hormetic-like effect”) was observed in both HepG2 and HEK293 cells in several of the BrdU ELISA assays. More research is required to determine if this effect represents a true indication of hormesis or is simply an artifact.

VI. Exposure to potassium or ammonium nitrate for a 24 hour period does not appear to have significant effects on the expression of PCNA, Hsp70, or Hsc70 in HepG2 or HEK293 cells. No changes were noted for VEGF expression in HEK293 cells treated with potassium or ammonium nitrate for 24 hours. Some subtle changes were noted for PCNA and Hsp70, but the implications of these changes are unknown.

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APPENDIX A:
Recipes for solutions used in analyses

Ponceau S Stain:
100mg Ponceau S
5mL glacial acetic acid
Make up to 100mL with distilled H_2O.
Mix well to combine.

Phosphate-Buffered Saline (PBS):
900mL distilled H_2O
0.23g NaH_2PO_4
1.15g Na_2HPO_4
9.0g NaCl
Mix until dissolved, then pH to 7.2-7.4. Autoclave if desired.

Phosphate-Buffered Saline plus Tween (PBST):
500mL PBS
500µL Tween 20

5% Milk in Phosphate-Buffered Saline plus Tween (5% milk/PBST):
500mL PBST
25g dry skim milk powder
Combine ingredients; stir well.

Coomassie Blue Protein Stain:
225mL distilled H_2O
225mL methanol
50mL glacial acetic acid
1.25g Coomassie Blue
Allow to mix overnight.

Coomassie Blue Destain Solution:
1.4L distilled H_2O
400mL methanol
200mL glacial acetic acid

SDS-PAGE Running Buffer:
144g glycine
30g Tris
10g SDS
2L double distilled H_2O

SDS-PAGE Transfer Buffer:
28.8g glycine
6.0g Tris
Add 1600mL of distilled H_2O & dissolve
Then add 400mL methanol

SDS-PAGE Loading Dye:
5mL 1.25M Tris HCl pH 6.8
20mL 10% SDS
10mL glycerol
10mL distilled H_2O
trace of bromophenol blue
Add 450µL SDS-PAGE Loading Dye to 50µL 2-mercaptoethanol and 500µL distilled H_2O before use.

Protein Extraction Buffer:
1mL 10mM Hepes pH 7.9
8mL 400mM NaCl
1.5mM $MgCl_2$
5mL glycerol
0.1mM EDTA pH 8
Mix and store at room temperature.

Just before use, add:
2µL/mL 2mM DTT stock
1µL/mL 10mg/L Aprotenin stock
1µL/mL 10mg/L Leupeptin stock
5µL/mL 0.5mM PMSF stock
Mix well, and keep on ice.

Cell Culture Medium:
For approximately 2L:
2 packets Minimum Essential Medium (MEM) powder (Gibco, #41500-034)
Add exactly 2L double-distilled H_2O
Allow to mix for at least two hours, then vacuum filter into sterile bottles.

Then add:
10mL of 1M sodium bicarbonate/phenol red solution per 500mL media
1% sodium pyruvate (5mL per 500mL media)
Store at 4°C until ready to use.

Immediately before use, add:
10% fetal bovine serum (50mL per 500mL media)
1% penicillin (10,000 units/mL)/streptomycin (10,000µg/L) (5mL per 500mL media)
After adding these components, store at 4°C.

10% SDS-PAGE Running Gel:
For 20mL:
7.9mL distilled H_2O
6.7mL 30% acrylamide mix
5.0mL 1.5M Tris, pH 8.8
0.2mL 10% SDS
0.2mL 10% ammonium persulphate
0.008mL TEMED (add just before pouring gels)

SDS-PAGE Stacking Gel:
For 5mL:
3.4mL distilled H_2O
0.83mL 30% acrylamide mix
0.63mL 1.0M Tris, pH 6.8
0.05mL 10% SDS
0.05mL 10% ammonium persulphate
0.005mL TEMED (add just before pouring gel)